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WASHINGTON, D. C., DECEMBER 28, 29 AND 30, 1937

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ABSTRACTS*

GENERAL BACTERIOLOGY

G1. A New Apparatus for the Determination of k Values of Strong Disinfectants. M. L. ISAACS, Columbia University, New York City.

An apparatus has been developed which permits contact of disinfectant and bacteria for as short a period of time as 0.2 second. It consists essentially of a pipette mounted over a platinum loop which in turn is placed a suitable distance above a revolving Petri dish containing water or other neutralizing agent. A known quantity of suspension is placed in the loop and a drop of disinfectant is allowed to fall from the pipette through the loop into the dish. The advantage of the method lies in the fact that it permits the determination of k values with such disinfectants as tincture of iodine and 5 per cent phenol without dilution. With *Staphylococcus aureus* at 35°C., the former has a value of 325 and the latter of 103. These figures mean that the former is capable of reducing 1×10^{163} power organisms to 1 survivor in the space of 0.5 minute, about the minimum contact period with ordinary methods used at present. The significance of this number may be partially appreciated when it is considered that it is over a quintillion quintillion times the number of *electrons* which could be packed side by side in a space the size of the universe. With the time of exposure used in the apparatus it is possible to have survivors even with the use of as few as 10,000 organisms per cubic centimeter.

G2. A Study of Mercury Antiseptics by the Agar Cup Plate Method. S. BRANDT ROSE AND RUTH E. MILLER, Philadelphia General Hospital and Woman's Medical College, Philadelphia, Pa.

Before undertaking the present experiments, the agar cup plate method was standardized. Using the standardized technique, four mercury antiseptics (mercury bichloride, mercurochrome, metaphen and merthiolate) were studied (1) in plain agar and (2) in horse blood

*This number of the JOURNAL has been edited by the Chairman of the Program Committee. Authors of the abstracts in the Scientific Proceedings have not seen proof, due to restrictions of time imposed by the printing schedule.

agar mixtures. The blood content of the test media varied from 1 to 50 per cent. A stock culture of *Staphylococcus aureus*, having the same resistance to phenol as the F. D. A. strain, was used.

All the mercury antiseptics showed a decreased zone of inhibition of bacterial growth as the blood concentration was increased. The results obtained were expressed by the formula

$$C = Ke^{-mz}$$

(C, concentration of blood; K, a constant for each antiseptic having the value of C when the zone is zero; e, base Napierian log; m, coefficient of inactivation of the antiseptic; z, zone in millimeters.)

Parallel studies in blood broth and blood agar mixtures showed (1) that the agar, *per se*, had no appreciable effect on the zone size and (2) that in general a correlation could be established between the ordinary antiseptic dilution procedure and the agar cup plate method.

68. *Some Factors Affecting the Germicidal Efficiency of Hypochlorite Solutions.* A. S. Rudolph and Max Levine, Department of Bacteriology, Iowa State College, Ames, Iowa.

Studies were made on the comparison of the germicidal efficiency of hypochlorite solutions containing 25, 100, and 500 p.p.m. available chlorine at constant temperature and initial reaction (pH). The effects of reaction and temperature were studied by using hypochlorite solutions containing 25 p.p.m. available chlorine and varying either the pH or temperature, keeping all other factors constant.

To 100-cc. portions of various hypochlorite solutions, contained in two-necked flasks which were held in a water bath at the desired temperature, was added 1 cc. of a freshly prepared suspension of the spores of *Bacillus metiens*. At appropriate intervals an amount of 5 cc. of the spore-hypochlorite mixture was transferred to 45 cc. of sterile distilled water, containing slightly more than enough sodium thiosulphate to neutralize the chlorine carried over. Dilutions were made and plated on standard nutrient agar. Counts were made after incubation for 24 hours at 30°C.

The results show that increasing the concentration of the available chlorine four-fold reduces the killing time to about one-half. Changes in pH affect the germicidal power of hypochlorite solutions to a much greater extent than do changes in the concentration of available chlorine, the germicidal power decreasing with decreasing hydrogen-ion concentration. Germicidal efficiency increased with rising temperatures. The killing time was reduced from 50 to 75 per cent for a rise of 10°C.

G4. A Technic for Preparing Bacterial Spore Suspensions of Uniform Resistance for Disinfection Studies. A. S. RUDOLPH AND MAX LEVINE, Department of Bacteriology, Iowa State College, Ames, Iowa.

Suspensions of spores of *Bacillus metiens* were prepared in Butterfield's dilution "C" water by scraping and washing the growth from standard nutrient agar cultures incubated for 20 days at 30°C. The suspensions were filtered through sterile filter paper to remove clumps and heated at 80°C. for 10 minutes to kill vegetative cells. These suspensions showed no appreciable change in resistance to a commercial calcium hypochlorite solution containing 1000 p.p.m. available chlorine during eight months storage in the refrigerator at 10°C. (50°F.), nor did the resistance differ significantly from that of dried spores suspended in powdered lactose.

G5. The Bactericidal Value of the Sterilamp. VIRGINIA L. ROSS AND W. L. MALLMANN, Department of Bacteriology and Hygiene, Michigan State College, East Lansing, Mich.

When non-spore-forming bacteria were exposed on agar plates at a distance of approximately 6 cm. to the emanations of a single Sterilamp, most species of pathogenic and non-pathogenic bacteria were killed in less than 2 minutes. The action of the emanations is a direct one and is not due to changes produced in the medium on which the organisms are placed. Mold spores showed practically no susceptibility to the light, but bacterial spores were very susceptible. The bactericidal powers of the Sterilamp do not appear to be any greater than other sources of ultra-violet light previously known.

G6. The Influence of Ultra-Violet Radiation upon the Heat-Resistance of Bacterial Spores. HAROLD R. CURRAN AND FRED R. EVANS, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

It is a well known fact that ultra-violet radiations lower the coagulation temperature of pure proteins. Quite as well established is the fact that death in microörganisms is coincident with, or rapidly succeeded by, coagulation of the cell protoplasm. Prompted by these considerations, we have studied the sporocidal action of various combinations of heat and light.

This work shows that bacterial spores may be sensitized to heat by preliminary exposure to ultra-violet light. Spore suspensions, exposed

to suitable ultra-violet radiation followed by heat at 98°C., undergo greater destruction than similar suspensions subjected to the same lethal factors applied in the reverse order. The increased mortality attributable to sensitization varies with the species and the extent of the killing influence. The sensitization mortality represents only a very small part of the total mortality, but in terms of the total number of survivors the comparative differences are significant.

The resistant spores in a culture are more susceptible to heat sensitization than are those which are easily killed by adverse influences. With both heat and ultra-violet radiation, a second application of the same treatment is more sporocidal than combinations of heat and light having the same killing influence. The heat sensitization of spores by ultra-violet radiation is observed when the spores are exposed in a dry or wet condition. Very short ultra-violet rays (1250–2000 Å) are found to be more effective in sensitizing spores to heat than are those which are transmitted by quartz.

G7. Studies on the Biological Effects of Supersonic Vibrations. PAUL J. BEARD AND W. F. GANTVOORT, Stanford University, Stanford University, Calif.

Apparatus for the generation of supersonic vibrations is described. It consists of an oscillator with two 500-watt tubes which can be used singly or in parallel. It follows in principle the apparatus described by Wood and Loomis, but with certain modifications. The wave length can be varied between 300 and 1000 kilocycles and the power can be adjusted by means of a variable resistance in the primary circuit. The energy is transmitted to a piezo-active quartz crystal submersed in a dielectric. The latter flows through a cooling system at a rate of 5 gallons per minute and the temperature can be held at any desired level.

On applying energy to the crystal the dielectric becomes so violently agitated that it builds up a fountain about 5 cm. in height. Objects suspended in this fountain are subjected to the effects of the vibrations.

Double-distilled water containing triple-distilled mercury developed, with 600 kilocycles, a turbidity lasting over several hours. Exposure for 4 minutes was lethal for mice. Exposure for 15 minutes reduced a culture of *Escherichia coli* from 70,000,000 to 20,000 cells per cubic centimeter.

G8. An Apparatus for Desiccating Stock Cultures. ALDEN F. ROE, Department of Bacteriology, Hygiene and Preventive Medi-

cine, School of Medicine, George Washington University, Washington, D. C.

An apparatus suitable for use in the laboratory with average facilities is described. It consists of a shielded Pyrex glass desiccator with an internal, cooled unit to maintain cultures in the frozen state during desiccation.

The cultures are first concentrated, then frozen with dry ice. After being prepared thus, they are placed in the cooling unit of the desiccator and the latter is evacuated and stored in a low temperature refrigerator for from 12 to 18 hours. Water vapor is absorbed by an efficient dehydrating agent, such as magnesium perchlorate. The water transfer is small, being approximately 10 cc. in the desiccation of 20 cultures.

The individual ampoules containing the desiccated suspensions (from 6 to 12 ampoules per culture) are evacuated in groups, sealed off, and are ready for storage. But a single ampoule need be opened when a culture is desired.

Data are presented on (a) the relative resistance of dried cultures to certain physical agents as well as (b) the value of different suspending media prior to drying.

G9. Further Observations on the Use of Sintered, Glass Filters in Bacteriological Work. HARRY E. MORTON AND E. J. CZARNETZKY, Department of Bacteriology, University of Pennsylvania School of Medicine, Philadelphia, Pa.

Until recently there were no filters available for bacteriological work which could be chemically cleaned as well as sterilized before use and which were adaptable to the filtration of small amounts of material. Sintered glass filters of No. 5 porosity presented these possibilities, if they could be relied upon to withhold bacteria. Recently the authors have described tests performed to ascertain the reliability of these filters in withholding bacteria. Since this report the filters have been employed in research and in teaching work for the filtration of solutions of soluble bacterial antigens, carbohydrates, etc., and have been found to be reliable in preventing the passage of bacteria. These filters can be used with very little loss for the filtration of small amounts of material.

G10. Studies on Bacterial Nutrition. A Comparison of Growth Factor Preparations. S. A. KOSER, R. D. FINKLE, A. DORFMAN AND F. SAUNDERS, University of Chicago, Chicago, Ill.

Attention was devoted chiefly to preparations or substances which, when supplied in minute amounts, cause development of some of the more exacting bacteria. The test substances were added in varying quantities to synthetic media. *Staphylococcus albus, Streptococcus hemolyticus* (scarlet fever), *Corynebacterium diphtheriae, Brucella abortus, Shigella dysenteriae* and, at times, *Saccharomyces cerevisiae* were used. These organisms failed to develop in synthetic media composed of amino acids, glucose and inorganic salts.

Certain fractions of extracts prepared by us from spleen, liver and yeast caused growth of 5 of the 6 organisms. Amounts of 0.3 microgram (gamma) per cubic centimeter of culture medium were at times sufficient to produce visible growth after several days. Larger amounts resulted in more rapid and luxuriant growth. The factor for propionic acid bacteria of Tatum, Wood and Peterson gave results similar to our preparations.

The following materials showed no growth-promoting effect when used singly under the conditions of our tests: the "sporogenes vitamine" of Knight, three plant auxins, phenyl-acetic acid, glutathione, *d l*-methionine, threonine, *d*-lysine, ascorbic acid, lactoflavin, adenine, uracil, allantoin, pimelic acid.

Nicotinic acid and vitamin B, together, but not separately, permitted growth of a staphylococcus, confirming Knight's work. Beta-alanine and nicotinic acid produced the same effect. Beta-alanine in a restricted synthetic medium permitted growth of a strain of diphtheria bacillus.

The growth-promoting effect could not be reproduced by adding small amounts of metals to the synthetic medium. Also, alterations in the physical character of the medium, particularly those designed to lower the oxidation-reduction potential, did not produce the growth-promoting effect.

G11. The Replacement of Meat Infusion by Known Substances in the Cultivation of Corynebacterium diphtheriae. J. HOWARD MUELLER, Harvard University Medical School, Cambridge, Mass.

Earlier work has shown that a considerable portion of the growth-inducing effect of meat extract for various strains of the diphtheria bacillus is due to the presence of nicotinic acid and β-alanine (or of carnosine) and of salts of potassium, magnesium and calcium. With certain strains, minute traces of pimelic acid, also presumably present in tissue extractives, show a definite growth-enhancing effect. With optimal quantities of these substances, however, only about two-thirds

of the amount of growth produced by whole meat extract can be obtained.

Further investigation has shown that by increasing the quantity of cystine added to the control medium and by supplying small amounts of salts of iron and manganese, even heavier growth is obtained than was formerly believed to be maximal. Thus, when estimating growth by the total nitrogen of the washed bacteria, about 2.25 mgm. of nitrogen were obtainable in the earlier work as against a supposed maximum of approximately 3.5 mgm. With the same strain of organism, and using the same basic medium (casein hydrolysate), 6.0 mgm. of nitrogen are now readily obtainable. It is probable, therefore, that no other organic constituent of meat infusion will be found to be essential for the strains used. That perhaps one or more additional inorganic substances will play a part is indicated by the slightly better growth obtained when a small amount of hay ash is included in the medium.

G12. An Improved Gelatin Hydrolysate Medium for Diphtheria Toxin Production. S. J. Johnson, A. M. Pappenheimer, Jr. and E. S. Robinson, Antitoxin and Vaccine Laboratory, Department of Public Health, Boston, Mass.

The recent studies of Mueller on the growth requirements of the diphtheria bacillus and our own on toxin production have led to the formulation of a simple medium for high-titer toxin production on a large scale. By using a complete acid hydrolysate of gelatin as a base supplemented by methionine, cystine, tryptophane, minute amounts of pimelic acid, β-alanine and nicotinic acid, the necessary salts, suitable concentrations of lactic acid, glucose and maltose and by regulation of the minute traces of iron, toxin can be produced routinely having an L_f value between 45 and 60 L_f per cubic centimeter. Over 600 liters of toxin averaging better than 30 L_f per cubic centimeter have been produced on a medium closely related to the above. The many advantages of this medium over previous formulas are as follows: (1) relatively inexpensive; (2) made rapidly and easily; (3) reproducibility excellent (no lot has fallen below 24 L_f per cubic centimeter); (4) quite specific for *Corynebacterium diphtheriae*, thus reducing the risk of contamination; (5) constant low nitrogen content (1.7 mgm. of nitrogen per cubic centimeter), thus toxoid formation, with only 0.2 per cent formalin, occurs in 4–5 weeks at 35°C. with no significant variation in the time necessary for complete detoxication; (6) absence of any significant amount of substance in the medium with higher molecular

weight than the simple amino acids, which makes it possible to obtain toxin that is about 70 per cent pure by means of a single ammonium sulfate fractionation and dialysis.

G13. Effect of Oxygen Tension on Site of Growth of Microörganisms, with Special Reference to Pathogenic Fungi. JOHN W. WILLIAMS, Massachusetts Institute of Technology, Cambridge, Mass.

It is probable that oxygen tension is important in the localization and invasion of pathogenic microörganisms. In test tube cultures of pathogenic fungi it is a factor determining depth of growth and invasion of the medium.

Certain pathogenic fungi, grown on Sabouraud's proof medium (1 per cent peptone, 4 per cent glucose, 1.5 per cent agar, pH 5.6) under atmospheric oxygen tension, show predominantly a surface growth; under 99.8 per cent oxygen a subsurface growth; and under 1 per cent oxygen, a sparser, limited, superficial, sometimes dry film-like growth. Removal of the latter type growth to atmospheric conditions often fails to provoke further growth. The organisms have apparently "asphyxiated" themselves. Similar fungi grown on a medium (0.25 per cent *d*-glutamic acid, 4 per cent glucose, 1.5 per cent agar, pH 5.6) producing a predominantly subsurface growth under atmospheric oxygen tension, grow more deeply in the medium under 99.8 per cent oxygen and predominantly on the surface under 1 per cent oxygen.

This work demonstrates that invasion of a medium is mutually dependent on constituents of the medium and oxygen tension. Invasion *in vivo* may likewise be dependent on constituents of the tissue and oxygen tension. Certain organisms invade the medium and grow on the surface using peptone (made from muscle) as the source of nitrogen, grow 0.4 cm. subsurface on substituting a hydrolysate of skin and almost 1 cm. below the surface on substituting a hydrolysate of hair. Variation in certain types of lesion in high and low altitudes substantiate further the importance of oxygen tension.

G14. Carbohydrate Fermentation Responses of the Aerobic Spore-Forming Bacilli. FRANCIS E. CLARK AND NATHAN R. SMITH, Bureau of Plant Industry, U. S. Department of Agriculture, Washington, D. C.

Further studies on the recently proposed taxonomic key for subgrouping the mesophilic, aerobic spore-bearing bacilli have led to an investigation of the carbohydrate fermentations of a large number of

cultures. These cultures were obtained in part from soil, and in part from various type culture collections, the latter supplying nearly a hundred differently named species. A synthetic agar medium, with monobasic ammonium phosphate as the source of nitrogen, and incubation periods of two weeks have been employed.

It is observed that those species which have been placed in the same sub-group because of other characteristics attack the same carbon compounds with but a few exceptions, and that the variations which do occur are usually limited to a few sugars. It is also observed that a single species frequently varies in its utilization of some one carbohydrate. Certain strains of *Bacillus megatherium* readily attacked mannose, while others did not; yet in subsequent tests with the mannose-negative strains, mannose-positive daughter colonies were obtained. Subcultures from these colonies attacked mannose as readily as the originally positive strains. Similar "slow" fermentations of certain carbohydrates are observed for other species.

From the data accumulated, it seems that the fermentation of carbohydrates under the above conditions is useful in the characterization of the various sub-groups of the mesophilic-aerobic bacilli, but that owing to the variations observed within the sub-group, it can not be used to establish the validity of a particular species.

G15. The Carbon Metabolism of Bacterium radiobacter. ALVIN W. HOFER, New York State Agricultural Experiment Station, Geneva, N. Y.

A study has been made of glucose utilization by four strains of *Bacterium radiobacter* in a mineral salts solution containing a low concentration (0.15 per cent) of sugar. After two weeks of incubation in Eldredge tubes at 25°C., the carbon dioxide was measured by titration of the barium hydroxide in the opposite arm of the tube. The amount of cellular material was estimated by centrifuging, drying and direct weighing of the cells. Glucose was determined by the Schaffer-Hartmann method. An attempt was made to measure the gum, but this was unsuccessful. It was found that 48–58 per cent of the glucose utilized was converted to carbon dioxide, 12–15 per cent to cells, and 30–38 per cent remained unaccounted for, probably because of conversion into gum. When incubated for 7 weeks, the amount of carbon dioxide produced was 65 per cent.

Figures for sucrose were very similar to those for glucose. Eighteen other carbon sources were tested simply by determining the percentage

of original carbon converted to carbon dioxide. This figure checked closely with the figures for glucose and sucrose, suggesting that the carbon metabolism of the organism is identical for the various carbon sources.

G16. The Bound Water Content of Vegetative and Spore Forms of Bacteria. C. A. Friedman and B. S. Henry, Department of Bacteriology, University of Washington, Seattle, Wash.

The authors have previously shown that the total water content of the vegetative cells and the spores of *Bacillus subtilis, Bacillus megatherium* and *Bacillus mycoides* is essentially the same. This finding confirmed the work of Virtanen and Pulkki which indicated that the observed heat-resistance of spores is not due to a low water content.

The present paper shows that the water existing in a free or unbound state, as determined by the cryoscopic method, is greater in the vegetative cells than in the spores of the three species of bacteria mentioned above. The theory is advanced that the bound water in the spores is not involved in the coagulation of the protein due to heat and that, while the total moisture content of the two types of cells is the same, the difference in free water might account for the observed difference in the heat-resistance of the cells.

G17. The Pigment of Bacillus violaceus. III. The Apparent Relation of Violaccin to Indigo. Walter C. Tobie, Mercedita, Puerto Rico.

It was shown by the author in 1936 that violaccin, the violet pigment of *Bacillus violaceus* (*Chromobacterium violaceum*), contains one or more pyrrolic groups in its molecule. Work on the culturing of the organism and extraction of the pigment has been suspended, but an examination of the small quantity of violaccin remaining on hand indicates that the pyrrolic group (or groups) occurs in a form similar to that found in indigo. Decomposition of the pigment by heating for several hours in a solution of sodium hydroxide in the presence of air gave a grape-like smell similar to that of anthranilic acid or its esters. Acidification with hydrochloric acid and filtration to remove amorphous brown material, gave a solution which, after careful diazotization with nitrous acid, produced the color reactions typical of anthranilic acid when treated with alpha-naphthol or dimethylaniline under the proper conditions. Due to the small amounts of pigment remaining, it was not possible to isolate anthranilic acid as such. Accordingly, the results must be

regarded only as a strong indication of the production of the acid or of some related compound. Since the pyrrolic product (previously obtained by strong reduction of violacein) is apparently not indol but a compound of higher molecular weight, and since the results of other workers indicate that the molecular weight of violacein is considerably greater than that of indigo, it seems highly probable that violacein may consist of an indigo nucleus with additional groups of considerable size attached.

G18. Classification and Pathogenicity of Microörganisms. III. Toxins and Toxic Products. ERNEST A. PRIBRAM, Loyola University Medical School, Chicago, Ill.

The "toxic products" of microörganisms attacking animal tissue are irregularly distributed among organisms of the same genus. A deeper study of their nature, however, reveals that the "toxic products" produced by species of related botanical units show common characteristics. In this abstract the term "toxic products" includes toxins with antigenic characteristics as well as substances which do not produce antibodies, but which are not simple chemical compounds.

The "toxic products" of microörganisms may be classified, as follows:

I. Hematotrope: a, erythrocytotoxins; b, granulocytotoxins; c, fibrinolytic toxins;
II. Lymphotrope: a, for lymph follicles of intestinal tract; b, for lymph nodes;
III. Histiotrope: a, for endothelium of vessels; b, for connective tissue and muscle fibers;
IV. Neurotrope: a, nerve cells; b, adrenals.

These "toxic products" have a distribution, as follows: *Micrococcus* Ia, b; *Streptococcus* Ia, b, c; *Vibrio* Ia; *Pseudomonas* (lipoids) Ia; *Escherichia* Ia, IIa; *Brucella* IIa; *Salmonella* IIa; *Eberthella* IIa; *Shigella* IIa, IVa; *Pasteurella* IIb, IIIa; *Hemophilus* IIIa; *Bacillus* Ia, IIIb; *Clostridium* Ia, IIIb, IVa, b; *Corynebacterium* IIIb, IVa, b; Fungi: *Eumycetes* (*Aspergillus* spores) IVa; *Basidiomycetes* Ia, IVa.

G19. Fixed Procedures and Tables for the Potency Estimation of Concentrated Staphylococcus Antitoxin. B. S. LEVINE, National Institute of Health, Washington, D. C.

The tables to be presented reduce procedures, calculations, and possibilities for error in the standardization of concentrated staphylococcus antitoxins to a minimum. The dilutions of the reagents, the kind of

animal employed in the hemolytic, dermonecrotic and lethal tests, and the methods and quantities of injection into the animals are embodied in the tables. Through the introduction of a dilution factor, $B = C_h/20$, the procedures for the dermonecrotic and lethal tests have been fixed for staphylococcus antitoxins of any concentration.

The calculation of results is prescribed by the following formulas:

$C_h = N_h \times V_h$, for the hemolytic test;

$C_r = N_r \times B \times V_r$, for the dermonecrotic test; and

$C_† = N_† \times B \times V_†$, for the lethal test.

C_h, C_r, and $C_† =$ the corrected and N_h, $(N_r \times B)$, $(N_† \times B)$ = the *non-corrected* numbers of antihemolytic, antidermonecrotic and antilethal units per cc. of the antitoxin being standardized.

$B =$ a dilution of the antitoxin such that 1 cc. contains 20 standard antihemolytic units. As stated above, $B = C_h/20$.

V_h, V_r and $V_† =$ factors of correction. These factors are functions of the *volume* of the U. S. Standard Staphylococcus Antitoxin which is indicated by the end-point of the control titrations.

Predetermined numerical values of N_r and $N_†$ and of V_r and $V_†$ in the above equations are entered into the tables to correspond to the end-points of the titrations, so that at the completion of the tests, the worker need only substitute such values for the symbols in the formulas and perform the task of multiplication.

G20. The Production of Staphylococcus Toxin in Fluid Media. E. P. CASMAN, Abington Memorial Hospital, Abington, Pa.

The addition of agar to the medium and cultivation under a gaseous mixture of carbon dioxide and oxygen have been two important procedures in the production of staphylococcus toxin. Small quantities of semi-solid medium are distributed in suitable containers to obtain a shallow layer and, after inoculation and incubation, the cultures are filtered to remove the agar. In order to facilitate the study of the factors involved in toxin production and for obvious practical considerations, elimination of the use of agar in the medium is desirable. It was found that by slowly bubbling the mixture of gases through the culture, good toxin production was obtained in from 48 to 72 hours, depending upon the pH, the composition of the medium and the gas mixture used.

G21. The Titration of Bifermentans Antitoxin and the Relationship between Clostridium bifermentans and Clostridium sordellii. SARAH E. STEWART, National Institute of Health, Washington, D. C.

In the course of the standardization of sordellii antitoxin the author was confronted with the problem of the relationship of *Clostridium sordellii* and *Clostridium bifermentans*. It is believed by various investigators that *Clostridium sordellii*, a proteolytic anaerobic bacillus which produces a strong exotoxin, and *Clostridium bifermentans*, a non-toxin-producing proteolytic anaerobe, may represent a single species.

For the titration of sordellii antitoxin, a dried toxin and antitoxin were prepared. The unit chosen was based on the one proposed by Alfredo Sordelli. Mice were used as the test animals and inoculations were made intrâvenously. Since *Clostridium bifermentans* does not produce a toxin, antisera were produced in rabbits against 4 strains and these antisera were tested against the dried sordellii toxin. The *Clostridium bifermentans* sera were found to give protection against 2–5 M.L.D. of sordellii toxin.

A comparative study was then made of strains from both of these species. They were found to be identical in morphology, colony formation and biochemical reactions. Cross-agglutination and cross-precipitation reactions were also obtained.

From the above studies, it appears that the two species are identical. Since the species name *bifermentans* has priority over *sordellii*, it is proposed that the designation *Clostridium bifermentans* should be used to cover the two species, and that the antitoxin made against the toxin be designated as *bifermentans antitoxin*.

G22. The Action of Chemical and Physical Agents on Clostridium welchii and Its Toxin. FRANCIS E. COLIEN, Creighton University, School of Medicine, Omaha, Neb.

The action of glutathione, cysteine, hydrogen peroxide, leucocytes and x-rays on the toxin of *Clostridium welchii* was studied *in vivo* and *in vitro*. The theories which have been given to explain the factors concerned with the destruction of toxin in cases of gas gangrene are various. Hence, an attempt was made to study *in vitro* the action on toxin of chemical substances found in living tissue. In addition, toxin, cultures of *Clostridium welchii* in broth containing sterile tissue, and toxin plus sterile tissue were subjected to x-rays. Pigeons were used in all cases to determine the resulting toxicity. The action of x-rays on guinea pigs, rabbits, roosters and pigeons injected with toxin or living organisms was also studied. Various methods were employed for inoculating the experimental animals with *Clostridium welchii*.

The possibility that certain chemicals, either alone or after release

from tissue by x-rays, act as detoxifying agents is discussed. We have little conclusive evidence on the detoxification of the toxin of *Clostridium welchii* by x-rays; there seems to be some evidence, however, that early treatment with x-rays may prevent the development of gas gangrene, and that it may even have some effect in treatment.

G23. A Stable Hemolysin-Leucocidin Isolated from β-Hemolytic Streptococci. E. J. Czarnetzky and Isabel M. Morgan, University of Pennsylvania School of Medicine, Philadelphia, Pa.

The isolation of a stable hemolysin-leucocidin from β-hemolytic streptococci has been reported. As hemolysin, it is active to a dilution of 1:40,000; it has been shown, by inhibition of the reduction of methylene blue by leucocytes, to be a leucocidin. The hemolysin has a molecular weight of 2260, and its crystalline derivative a molecular weight of 720. Further work has shown the empirical formula of the stable hemolysin to be $C_{75}H_{148}O_{71}P$.

Intraperitoneal injection of the crystalline derivative into mice (Dr. H. Molitor) has shown that the minimal lethal dose for mice is of the order of 0.001 mgm. By the injection of single doses of various sizes into rabbits, we have found the minimal lethal dose to be of the order of 1 mgm. Repeated injection into rabbits of sub-lethal doses (of increasing size, approaching the lethal dose) produced no quantitative effect on the circulating leucocytes; however, an anemia developed with an erythrocyte count of about 3.5 million per cubic millimeter, compared with a count of about 5.5 million per cubic millimeter in a group of normal rabbits living under the same conditions. The rabbits did not develop a tolerance for the material, the final fatal dose in each case being of the same order as the minimal lethal dose for normal rabbits (the material has never been shown to be antigenic in the sense of producing antibodies on injection).

G24. The Antigenic and Synergistic Action of a Toxic Serum Extract of Hemolytic Streptococci. Mark P. Schultz and Edythe J. Rose, National Institute of Health, Washington, D. C.

The experiments reported concern the antigenic and synergistic capacities of a toxic serum-extract of hemolytic streptococci (described by Weld). A special study was made of the ability of this toxic serum-extract to cause an animal's own serum to become irritating when injected into its peculiarly hypersensitized skin. The toxic serum-extract was prepared by shaking portions of pooled rabbit serum for one

hour at room temperature with sediment from broth cultures of a Group A hemolytic streptococcus (Strain C-203). The same strain was also used for animal inoculations and serological tests. Five groups of rabbits were studied: Group A, untreated control group; Group B, culture given intravenously; Group C, culture given intracutaneously; Group D, toxic serum-extract given intravenously; and Group E, toxic serum-extract given intracutaneously. Humoral antibodies for the homologous organism appeared in high titer in the sera from members of Group B, and irregularly in those from Group C, but not in other groups. The response to intracutaneous injection of culture was altered only in Groups B and C; an immune hypoergic reaction was elicited in the former, and a hyperergic response was demonstrable in the latter. Cutaneous hypersensitivity, both to the toxic extract *and to normal untreated rabbit serum*, developed in members of Group E and to a lesser degree in those of Group C. Such cutaneous lesions in Groups B and D were smaller than among the controls. The toxic serum-extract was most effective as a synergist in inducing cutaneous hypersensitivity in rabbits to normal (untreated) blood serum of that species.

G25. Demonstration of Bacterial Anticoagulants in vivo. ERWIN NETER, University of Buffalo and Children's Hospital, Buffalo, N. Y.

Of the two substances, fibrinolysin and anticoagulant, produced by the hemolytic streptococcus *in vitro*, the former has previously been demonstrated *in vivo*. Experiments are reported herewith which deal with the demonstration of bacterial anticoagulants in purulent exudates of lesions in man. The tests were performed by mixing the supernatant fluid of the exudate with plasma and a solution of calcium chloride. The anticoagulant continuously inhibited plasma coagulation.

Of 109 purulent exudates, 25—including empyema fluids, peritoneal and pericardial exudates, spinal fluids and abscess material—contained anticoagulants. The microörganisms implicated were: *Streptococcus hemolyticus*, *Streptococcus fecalis*, pneumococcus, staphylococcus, influenza bacillus, *Escherichia coli* and *Clostridium welchii*. These experiments show that anticoagulants may be found in exudates of man due to a variety of microörganisms.

In view of Goodner's observations that the edema fluid from dermal pneumococcal lesions in rabbits retards blood coagulation, pneumococcal exudates of man were tested for the presence of anticoagulant. Of 29 exudates, 3 due to pneumococcus (Types I, VII and XIII) were positive.

The nature of the anticoagulating factor found *in vivo* is not known.

It may be mentioned, however, that the anticoagulating action of purulent exudates may be due to at least two different mechanisms, because it was possible to inhibit the anticoagulating activity in some exudates (6 specimens) by means of normal horse serum and in others (4 specimens) by means of a 0.25 per cent solution of calcium chloride.

G26. The Systematic Relationships of the Autotrophic Bacteria. R. S. Breed and H. J. Conn, New York State Agricultural Experiment Station, Geneva, N. Y.

The systematic relationships of autotrophic bacteria remain an interesting problem for speculation. The assumption that such bacteria represent the most primitive known forms of life was questioned by the authors in 1918. It is possible that these forms represent a specialized adaptation to inorganic food developed after certain types of bacteria had become adapted to life in soil. Bacteria with their highly complex protoplasmic structure can hardly be regarded as the simplest forms of life, even though their metabolism is simple in character. Research has shown that organized bodies exist which are apparently living but which have a much simpler protoplasmic structure than autotrophic bacteria.

Recent discussions of the systematic relationships of bacteria (Pribram, Kluyver and Van Niel, Rahn) have clarified the relationships of autotrophic and other groups of bacteria in a helpful way, but the discussions are not in entire accord with each other nor in complete agreement with the ideas expressed by others.

The autotrophic organisms, in recent editions of Bergey's Manual, have been placed in the family *Nitrobacteriaceae*, distinct from either *Bacteriaceae* or *Coccaceae*. As given, the family also contains certain parasitic and saprophytic bacteria (*Rhizobium*, *Azotobacter* and *Acetobacter*) which should be removed from the group. The present tendency not to recognize divisions on the basis of cell-form, makes it possible to consider the autotrophic bacteria as a family or tribe coördinate with the tribes of the present family *Bacteriaceae.* If the family *Bacteriaceae* is dropped, the tribes become families.

G27. Taxonomic Relationship of Lactobacillus bifidus [Bacillus bifidus (Tissier)] and Bacteroides bifidus (Eggerth). James E. Weiss and Leo F. Rettger, Department of Biology, Brooklyn College, Brooklyn, N. Y. and Department of Bacteriology, Yale University, New Haven, Conn.

A taxonomic study was made of various strains of bacteria which

had been reported by several authors under the species name, "bifidus." These strains included *Bacterium bifidum* (Orla-Jensen), *Bacteroides bifidus*, Groups I and II (Eggerth) and *Lactobacillus bifidus* (Weiss and Rettger). It was shown (in full agreement with Eggerth) that there were at least two types of bifidus-like organisms, both members of the *Lactobacillus* genus. One is the true *Lactobacillus bifidus*, Type I, the original "*B. bifidus*" of Tissier. Eggerth's *Bacteroides bifidus*, Group I, is identical with this organism. The other type is the so-called *Bacteroides bifidus*, Group II (Eggerth), which is apparently identical with *Bacterium bifidum* of Orla-Jensen; for this type the authors propose the name *Lactobacillus bifidus*, Type II, or *Lactobacillus parabifidus*. These two types have much in common, but differ in certain important respects, particularly in their action on mannose, xylose, melizitose and arabinose. They also differ in their oxygen requirements, continued ability to branch, colonial form, serological relationships, and in the amounts and types of acids formed.

The organisms group themselves as follows:

Lactobacillus bifidus, Type I. Synonyms: *Bacillus bifidus* (Tissier); *Bacteroides bifidus*, Group I (Eggerth); *Lactobacillus bifidus* (Weiss and Rettger).

Lactobacillus bifidus, Type II, (*Lactobacillus parabifidus*). Synonyms: *Bacteroides bifidus*, Group II (Eggerth); *Bacterium bifidum* (Orla-Jensen).

G28. Comparison of Bacterium necrophorum from Ulcerative Colitis in Man with Strains Isolated from Animals. G. M. Dack, L. R. Dragstedt, Robert Johnson and N. B. McCullough, Department of Bacteriology and Parasitology, University of Chicago, Chicago, Ill.

A study has been made of 42 strains of non-sporulating Gram-negative anaerobes which we have called *Bacterium necrophorum*. Nineteen of these strains were isolated from the colons of patients with chronic ulcerative colitis. According to the literature some of these strains should be named *Bacterium funduliforme* and others *Bacterium necrophorum*. Twelve strains isolated from bovine liver abscesses would qualify as representatives of the latter group.

The purpose of this investigation was to compare the two groups in order to determine whether we were dealing with one or with two species. Accordingly, a study was made of the growth requirements of the strains, colony morphology, cell morphology on different media,

biochemical reactions, pathogenicity for rabbits and ability to produce ulceration of the colons of experimental animals. From this study no sharp line differentiating these bacteria was found. Strain variations in morphology were observed, but these were not sufficient for species differentiation. Likewise there was a difference in pathogenicity; only the 12 strains isolated from bovine liver abscesses were lethal to rabbits when injected subcutaneously. However, there were marked variations among the other strains in regard to ability to produce lesions. The type of lesion appeared to be similar with all strains, with a gradient from slightly virulent to highly virulent types, as represented by the strains isolated from the bovine liver abscesses.

Since there is no clear cut method for separating these two species, it appears better to retain the name, *Bacterium necrophorum*.

G29. *Nitrogen Availability as an Aid in Differentiation of Bacteria in the Coli-Aerogenes Group.* N. B. Mitchell and Max Levine, Department of Bacteriology, Iowa State College, Ames, Iowa.

The availability of the nitrogen of nucleic acid and certain of its degradation products for the coli-aerogenes group of bacteria was investigated. The purpose was to secure an approach, other than by carbohydrate studies, to the systematic and physiological relationships of the organisms comprising this group. Previous work on uric acid suggested the possibility of employing the purines for this purpose.

The compounds tested included yeast nucleic acid, xanthine, adenine sulfate, uric acid, uracil, allantoin, hydantoin and urea. Over 350 coli-aerogenes strains were used. This collection was considered representative of the *Escherichia, Aerobacter* and "intermediate" groups.

Xanthine and adenine were attacked by all strains, and consequently had no differential value. The *Aerobacter* strains utilized the nitrogen of all the other compounds, whereas the *Escherichia* and "intermediate" strains were each able to utilize the nitrogen of only one of the compounds listed. Uracil was an available nitrogen source for *Escherichia*, but not for "intermediate" strains. Urea was an available nitrogen source for "intermediate," but not for *Escherichia* strains. These data, when correlated with the results of Voges-Proskauer, citrate, hydrogen sulfide, and indol tests, strengthen the evidence for allocating the "intermediate" strains to a separate genus, *Citrobacter*. About 90 per cent of the "intermediates" gave reactions identical with those of a transfer of the original strain, *Citrobacter freundii*, the type species of the genus *Citrobacter*.

Studies of nitrogen availability provided a means for generic allocation of a number of strains which could not be adequately classified on the basis of carbohydrate dissimilation.

G30. A Study of the Paracoli Group. JACOB L. STOKES, R. H. WEAVER AND M. SCHERAGO, Department of Bacteriology, University of Kentucky, Lexington, Ky.

Thirty-two strains of the paracoli group were isolated from human feces and their morphological and biochemical characteristics were studied. All strains produced some degree of fermentation of lactose on continued cultivation in 5 per cent lactose broth. From 22 strains, variants were obtained that were able to ferment 1 per cent lactose broth within 48 hours with the production of acid and gas. These variants, therefore, were indistinguishable from members of the coli-aerogenes group.

It is concluded that the strains of the paracoli group studied are variants of various members of the coli-aerogenes group. On the basis of the methyl-red, Voges-Proskauer and sodium citrate tests, 30 of the strains appear to be variants of members of the *Escherichia* genus and 2, variants of members of the coli-aerogenes "intermediate" group.

G31. Comparative Studies of Methods for the Detection of Hydrogen Sulfide in the Coli-Aerogenes Group. CHARLES A. HUNTER AND JAMES E. WEISS, State Health Laboratory, University of South Dakota, Vermillion, S. D., Kansas Public Health Laboratories, Topeka, Kan., and Department of Biology, Brooklyn College, Brooklyn, N. Y.

The ability of the coli-aerogenes group to produce hydrogen sulfide was studied, using 152 strains. Ninety-eight cultures were isolated from water supplies, 35 from human feces and 19 were stock cultures. The cultures were classified into three groups: *Escherichia coli*, *Aerobacter aerogenes* and "intermediates." The methods for detecting the formation of hydrogen sulfide were: Difco lead acetate, Difco peptone iron, semi-solid peptone iron (0.5 per cent agar), semi-solid bismuth mannitol agar (Hunter and Crecelius) and bismuth filter paper. Of the 78 cultures of *Escherichia coli*, only 2 gave a positive reaction in the lead acetate and peptone iron media, but in the bismuth medium all 78 were positive, and with bismuth paper 57 were positive. With the 16 aerogenes strains no hydrogen sulfide was formed in the lead acetate or peptone iron media, while all strains were positive in the bismuth

medium and 12 gave positive reactions with bismuth paper. There were 57 cultures classified as "intermediates," of which 19 were positive in the lead and iron media and 56 were positive with semi-solid bismuth mannitol agar. The bismuth paper showed that 34 cultures produced hydrogen sulfide. The semi-solid peptone iron medium failed to show any increase in the number of positive reactions and did not prove as satisfactory as the usual 1.5 per cent agar. These results show that practically all members of the coli-aerogenes group produce hydrogen sulfide, when tested with a sensitive indicator, the only differences in the organisms being the quantity of hydrogen sulfide produced.

G32. The Morphological Variation of the Tubercle Bacillus. HARRIETTE D. VERA AND LEO F. RETTGER, Department of Bacteriology, Yale University, New Haven, Conn.

The cellular variation of the tubercle bacillus was studied in microculture by means of hanging block preparations which permitted observation of single cells or microcolonies over considerable periods of time. Stained smears, impression preparations, filtration experiments, and cross sections of colonies were also used. An attempt was made to correlate acidfastness and growth conditions with the forms observed.

Morphological variants were induced readily and in great diversity by alteration of food supply and oxygen tension. They included club forms, spore-like bodies, granules of various sizes, coccoid, diphtheroid, and branching cells. Formation of granules in, or from, rods was observed repeatedly. Granules, "spores," and all cells that differed greatly from the typical rod failed to grow under conditions favorable to multiplication of bacilli. Branched cells, however, were observed to grow and segment so as to form bacilli indistinguishable from neighboring bacilli formed by simple division. Although branching was observed in the four strains studied, the usual method of reproduction was division. No evidence of reproduction by granular or filtrable forms was obtained.

G33. Notes on the History of Bacteriology. The Introduction of Agar-Agar into Bacteriology. ARTHUR PARKER HITCHENS AND MORRIS C. LEIKIND, Army Medical School, Washington, D. C. and Institute of the History of Medicine, Johns Hopkins University, Baltimore, Md.

Agar-agar was introduced into bacteriology by Frau Fanny Eilshemius Hesse, wife of Dr. Walther Hesse, a district physician, of Schwar-

zenberg, Saxony. Although no exact date is ascertainable, agar was first used sometime during the early 1880's. Frau Hesse who was assisting her husband in his studies on the bacterial content of the air (begun in Koch's laboratory) suggested the use of agar as a solidifying agent to eliminate the difficulties arising from the use of gelatin. The idea apparently came to Frau Hesse from her use of agar in her kitchen in the making of fruit jellies. She had been given the recipe by her mother who in turn had received it from a Dutch family; they had brought it from Batavia in the Dutch East Indies, where they had lived before coming to America.

When agar was found to be successful in Dr. Hesse's experiments, he communicated the discovery to Robert Koch by letter. Koch recognized the value of this and adopted its use in his laboratory. No formal paper was ever published on this discovery.

Frau Hesse of German (Hanover) descent was born in Jersey City, N. J., in 1850. She met her husband while travelling in Germany and spent the rest of her life there. She died in 1934, in Dresden.

MEDICAL BACTERIOLOGY, IMMUNOLOGY AND COMPARATIVE PATHOLOGY

M1. Antigenicity, with Especial Reference to Infectious Agents. MICHAEL HEIDELBERGER, College of Physicians and Surgeons, Columbia University, and Presbyterian Hospital, New York City.

A brief discussion is given of what is known of the chemical basis of the immunological specificity of single antigens, such as crystalline egg albumin, crystalline serum albumin, and thyroglobulin. The multiplicity of antigens in most natural products and infectious agents is emphasized. An account is given of some of the chemical factors involved in the immunological behavior of pneumococci, streptococci, tubercle bacilli, and the *Salmonella* and cholera groups of microörganisms.

M2. Changes which Occur in Antigens in the Animal. LASZLO DETRE, National Institute of Health, U. S. Public Health Service, Washington, D. C.

The virulence-enhancing effect of animal passage is well established. No investigations, however, have been made on the rôle of the different organs in the production of the antigenic changes correlated with the change in virulence. Experiments undertaken partly with *Bacillus typhosus* and partly with a newly isolated paratyphoid strain (Stanley)

showed the previously unknown fact that cultures isolated from different organs may show a difference in antigenic structure. For the Stanley strain studied, it was found that the blood cultures are the richest in O and poorest in H antigens, whereas the liver cultures are the richest in H and the poorest in O antigens.

In vitro and *in vivo* experiments with live and protein antigens made in order to elucidate changes occurring in them *in vivo* gave results, as follows. Proteins, when diluted with fresh blood or fresh active serum, give strong evidence of gradual disintegration and splitting. They show (1) higher toxicity when injected intradermally; (2) their dialysates through proper membranes give good precipitation with protein precipitins of high titer; (3) their dialysates give the normal ink reaction, as described by the author.

Analogous results can be obtained with blood drawn from animals shortly after intravenous injections with different protein antigens.

Inactive serums or even saline act similarly, but in a slighter degree. The precipitation effect with dialysates shows peculiarities which differentiate them from the usual precipitation tests.

An approach for the better understanding of both the Schwartzman and Foshay phenomena is undertaken.

M8. The Labile Antigen of Streptococcus pyogenes and Its Derivatives "M," "C" and Filtrate-Streptolysin. Stuart Mudd, E. J. Czarnetzky, David Lackman and Horace Pettit, University of Pennsylvania School of Medicine, Philadelphia, Pa.

The labile antigens of hemolytic streptococci of Lancefield Group A have been shown to be the dominant surface antigens of virulent strains, and have been identified with Griffith's type-specific agglutinogens. Lancefield's type-specific hapten "M", her group-specific hapten "C" and the oxygen-labile streptolysin found in culture filtrates have all been shown to be derivatives of labile antigen. Substances "M" and "C" may be prepared by acid hydrolysis of labile antigen and fractionation of the hydrolysate.

Antisera prepared in rabbits by the injection of dead followed by living streptococcal cells contain separate antibodies against labile antigen and its derivatives. Antibodies against "M" and "C" may be completely precipitated from such antisera without appreciably affecting the titer of the antibody against labile antigen. Antiserum produced by injection of pure labile antigen reacts only with labile antigen and not with "M", "C" or filtrate-streptolysin. Saturation of strep-

tococcal antiserum with labile antigen, on the other hand, removes antibodies not only against labile antigen, but also against the derivatives "M", "C" and filtrate streptolysin.

The complete labile antigen reacts with antibodies against itself and its derivatives, the derivatives react only with antibodies against themselves and not with antibody against the complete antigen. These results may have somewhat general interest in view of the growing evidence to suggest that many of the fractions hitherto isolated from bacteria may be derivatives of a smaller number of more complex and labile molecules.

M4. Antigenicity of the Friedländer Group. LOUIS A. JULIANELLE, Oscar Johnson Institute and Department of Bacteriology, School of Medicine, Washington University, St. Louis, Mo.

A study of the antigenic relationships of the encapsulated Gram-negative rods has been made in the past by means of the agglutination reactions of the encapsulated organisms in homologous and heterologous sera. These observations have already been reported in a number of communications. The studies have been continued to include an analysis of the species-specific or R antigens of the Friedländer, rhinoscleroma, ozaena, aerogenes, and granuloma organisms. To a lesser extent, observations have also been made on a few representative strains of *Bacterium coli*. The results of the latter reactions indicate that the encapsulated organisms commonly classified as the Friedländer group fall into two large classes as determined by the group-specific antigen, all strains of true Friedländer bacilli comprising the one, and all strains of rhinoscleroma, ozaena, aerogenes, and granuloma organisms falling into the other. Both groups in turn differ from both the type-specific and species-specific antigens of the colon group.

M5. A Comparison of the Precipitation Reaction in Immune Serum Agar Plates and the Protection of Mice with Meningococcus Antiserum. MARGARET PITTMAN, SARA E. BRANHAM AND ELSIE M. SOCKRIDER, National Institute of Health, Washington, D. C.

A number of therapeutic polyvalent meningococcus antisera have been studied by two methods: (1) precipitation reaction of type-specific meningococci in agar plates containing immune serum and (2) the protection of mice against highly virulent meningococci in a solution of mucin.

With the sera tested, it was observed that those sera which gave an

intense halo would, in high dilution, protect mice against many minimal fatal doses of meningococci of the type homologous to that producing the halo; i.e., as little as 0.0005 cc. of serum protected against 500,000 or more fatal doses. On the other hand, if the halo were faint or absent, the mice were never protected against more than a few minimal fatal doses, even when large amounts of serum were given.

With meningococci of the I–III group, all sera produced a precipitation reaction to some degree. But with Type II cultures, only an occasional serum produced a halo and it was never intense.

The significance of these results is discussed.

M6. The Antigenic Qualities of Vaccinia Virus. J. Craigie and F. O. Wishart, Connaught Laboratories and School of Hygiene, University of Toronto, Toronto, Canada.

The development of immunity in rabbits to infection with vaccinia virus is associated with the appearance of a number of humoral antibodies: (a) neutralizing antibodies, (b) L, S and X agglutinins, (c) L and S complement-fixing antibodies and (d) L and S precipitins.

The variation in immunizing qualities shown by different lots of killed elementary bodies or virus-free gradocol filtrates of fresh soluble antigen suggests a high degree of lability of the antigenic qualities required to produce immunity to vaccinia virus. Two labile antigens are demonstrable *in vitro;* these are the X and L agglutinogens. The latter, in association with the S antigen, dissociates from the elementary bodies and, in solution, gives a specific precipitin reaction. On the other hand, a precipitin reaction referable to the X agglutinogen has not been clearly demonstrated.

As previously reported, the L antigen occurs in a state of combination with the S antigen. It has since been found that on prolonged storage at from 4° to 8°C. this combination may dissociate into separate L and S fractions prior to ultimate inactivation of the L antigen. The L and S antigens produce antibodies related neither to immunity nor to the neutralizing antibody. The L antigen is inactivated at 56°C., but the S antigen retains its antigenicity when heated to higher temperatures. Treatment of the latter antigen with N acetic acid at 100°C., however, impairs its antigenicity but not its precipitability with antibody.

M7. On the Antigenic Properties of Poliomyelitis Virus. E. W. Schultz, Stanford University, Stanford University, Calif.

It is generally known that the virus of poliomyelitis stimulates the

formation of neutralizing antibodies. It does this not only in man and susceptible monkeys, but also in individual animals which are naturally refractory to this disease.

The nature of the antigen-antibody reaction is not fully understood. Experimental results indicate that neutral mixtures can be dissociated and the virus rendered infectious again. The reaction of antigen and antibody does not appear to be associated with the fixation of complement and a specific flocculation has not been demonstrated.

Evidence that the virus possesses sensitizing properties has not been elicited. Infected and convalescent monkeys show neither a specific skin reaction nor a temperature rise after reinjection with large amounts of active virus. Tests for sensitization by the Schultz-Dale method, using uteri of convalescent monkeys, have yielded negative results, as have also attempts to induce sensitivity actively or passively in guinea pigs.

Extensive studies to elicit a way of identifying the presence of either the virus or the antibody without the use of monkeys have yielded negative results.

While injections of virus stimulate the formation of neutralizing antibodies, true immunity to infection seems to result only after the virus has come into intimate contact with nerve cells and produced a specific change in them. Such acquired resistance is not always associated with demonstrable antibodies in the blood. This indicates that the reaction is determined by the tissue on which the virus acts.

There is evidence that some strains differ in antigenic constitution. The practical implications of this antigenic variation are apparent.

M8. Some Effects of Formaldehyde on Horse Antipneumococcus Serum and Diphtheria Antitoxin and Their Significance for the Theory of Antigen-Antibody Aggregation. HARRY EAGLE, Johns Hopkins Medical School, Baltimore, Md., and the U. S. Public Health Service, Washington, D. C.

Small amounts of formaldehyde inhibit the precipitating activity of horse diphtheria antitoxin with toxin and of horse antipneumococcus serum with the homologous capsular carbohydrate. Approximately 1 part of commercial formaldehyde to 500 parts of serum, acting for 24 hours, inhibits the flocculating activity completely. In both cases, the combining affinity of the treated antibody for the corresponding antigen is unaffected, as determined both by *in vitro* experiments and by animal protection. More intensive treatment of the antipneumococcus

serum causes an apparent loss of its bacterial agglutinating activity, but on centrifugation the organisms cohere: combination has occurred, and only the spontaneous aggregation is prevented. On sufficient treatment, all antibody activity is destroyed. The quantitative relationships suggest that only a few groups in the antibody molecule need be modified by formaldehyde in order to prevent aggregation. Qualitatively, these are the same effects produced by diazo compounds, as described previously.

That aggregation is prevented by procedures which do not demonstrably effect the ability of the antibody to combine with antigen suggests that the secondary aggregation of antigen-antibody compounds is a non-specific phenomenon not primarily determined by residual specific combining groups in the bound antibody. The amount of formaldehyde which just suffices to prevent aggregation also causes an increase in the solubility of pneumococcus antibody, so marked as to suggest that the loss of precipitating activity is actually due to the increased solubility. This supports the hypothesis that the primary cause of specific antigen-antibody aggregation is the relative insolubility of the bound antibody.

M9. Intradermal Tests in Leprosy with Antigens of Acid-Fast Bacteria.
Earl B. McKinley, Department of Bacteriology, Hygiene and Preventive Medicine, School of Medicine, George Washington University, Washington, D. C.

The results of 5174 skin tests performed in the Philippine Islands on leprosy cases and controls with various antigens prepared from acid-fast bacteria are reported. The antigens included a protein, a polysaccharide, a phosphatide, a wax, and leprosinic acid from the wax, prepared from a supposed culture of *Mycobacterium leprae* (Hygienic Laboratory Strain No. 370) by Dr. R. J. Anderson. In addition, a number of "TPT" antigens (tuberculin-protein-trichloracetic-acid-precipitated), as prepared by Dr. E. R. Long, were available. Such proteins from *Mycobacterium tuberculosis* (human, avian and bovine strains), *Mycobacterium smegmatis, Mycobacterium phlei, Mycobacterium marinum,* a so-called *Mycobacterium murium,* and proteins from supposed cultures of *Mycobacterium leprae* of Duval, Daines, Karlinski, and a strain isolated in Philadelphia, were tested. Serological relationships between these antigens and proteins from other acid-fast bacteria have been demonstrated by Henderson.

These antigens were tested in early and advanced cases of leprosy;

in bacteriologically negative cases being released on parole; in the children of lepers removed from the leper colony from 6 to 24 months after birth; in family groups, i.e., the relatives of lepers; in normal children without previous contact with leprosy; and in professional contact groups of physicians, nurses, etc., at Culion.

An analysis of the tests shows that none is specific for leprosy. A skin test comparable to the tuberculin test is much needed as an aid in the early diagnosis of leprosy, but this study indicates that no such test is yet available.

M10. A Shock Reaction in the Monkey. I. Dosage. LENORE M. KOPELOFF AND NICHOLAS KOPELOFF, Department of Bacteriology, N. Y. State Psychiatric Institute and Bureau of Laboratories, N. Y. City Department of Health.

Previously we have shown that the monkey (*Macacus rhesus*) can be sensitized and specifically shocked with undiluted hen's egg-white. In the present study it was found that the minimum single sensitizing dose was approximately 0.2 cc. per kilogram of body weight. Doses ranging from 0.001 to 0.35 cc. per kilogram were tested on 20 monkeys. A standard shocking dose of 4.6 cc. per kilogram was used after an interval of 4 weeks.

The optimum shocking dose was in the range of 2.3 to 4.6 cc. per kilogram. Of 26 monkeys, 22 collapsed or died after the above dosages. Forty normal monkeys injected with the same amount of antigen showed no ill effects. An interval of 4 weeks between sensitization and shock proved almost twice as effective as an interval of 3 weeks. When multiple sensitizing doses were employed, the shocking dose could be considerably reduced. Sensitivity was found to persist for from 6 to 8 months in 5 of 9 monkeys tested.

Precipitin tests were made on practically all the monkeys studied and in general proved negative. Only when multiple sensitizing doses were used did the serum show the presence of precipitins (low titer). However, passive transfer of anaphylaxis with such serums to guinea pigs was usually successful.

A long series of subcutaneous injections produced a typical Arthus phenomenon in the skin of monkeys, in 5 with egg-white and in 2 with horse serum.

Since we have not yet fulfilled all the criteria for anaphylaxis, it would be more desirable at this time to regard the phenomenon described as a shock reaction.

M11. Influence of Artificially Induced Fever on Specific Antibody Production in Rabbits. HAROLD V. ELLINGSON AND PAUL F. CLARK, Department of Bacteriology, University of Wisconsin Medical School, Madison, Wis.

By the use of an artificially heated chamber, with suitable control of temperature and humidity, the febrile state has been maintained in rabbits at various levels and for various periods during immunization or infection. Each series of animals has included 12 individuals, 6 being thus subjected to artificial fever, and the remaining 6 being kept at room temperature.

Three series of animals were immunized with sheep erythrocytes. In two of these series, severe fevers around 41.7°C. (107°F.) were induced in test animals; antibody responses in these animals were noticeably lower than in the case of the controls. In one series, relatively mild fevers around 40°C. (104°F.) were induced; here there was no appreciable difference between the antibody responses of test and control animals. In two series immunized with a *Bacillus typhosus* vaccine, the test animals subjected to severe fevers showed a less vigorous antibody response than did the controls. In a series immunized with recrystallized egg albumin, the test animals showed a noticeably lower antibody response than did the controls. Living, virulent pneumococci were injected intracutaneously into a series of rabbits. Among those exposed to severe fever during the early stages of the ensuing infection, the process seemed more severe than among the controls, as indicated by a more rapid development of a fatal septicemia, and by a smaller number of survivors.

These experiments suggest that severe fever, as induced under the conditions of this work, actually impairs the immune responses of the rabbit.

M12. Factors Influencing the Production of Guinea Pig Complement of Satisfactory Titer. J. E. FABER, JR. AND L. A. BLACK, Department of Bacteriology, University of Maryland, College Park, Md.

The influence of age, weight, sex, litter mates, feed and shelter of guinea pigs upon the hemolytic activity of their complements was studied.

Young guinea pigs consistently had low, unsatisfactory titers. All males gradually increased in titer between the 3rd and 6th month, while females almost invariably had a lower titer than males after the 4th,

5th or 6th month due to pregnancy. This effect of pregnancy overshadowed the influence of other factors. Males did not usually attain a titer of 0.1 cc. of a 1:10 dilution until reaching an age of from 5 to 7 months, and 57 per cent did not reach this titer until weighing 600 grams or more. Variation occurred in females, both within the same litter and between different litters, although in limited observations on males the titers within the same litter agreed rather closely, with the usual differences between litters.

Maintenance of guinea pigs for more than a year upon one of three commonly used rations indicated that any satisfactory diet had little effect upon complement, although males reached a good titer earliest upon a complete commercial feed supplemented by cabbage every other day. The titer of guinea pigs reared and kept outdoors was not affected by extreme cold in winter, nor by heat in summer. However, during their second summer, many mature animals died from suffocation during periods of excessive heat and humidity.

M18. The Relation of Virulence to the Course of Salmonella aertrycke Infection in Mice. R. M. PIKE AND G. M. MACKENZIE, The Mary Imogene Bassett Hospital, Cooperstown, N. Y.

The experiments attempt to demonstrate how virulence modifies the course of *Salmonella aertrycke* infection in mice. Cultures of low, of intermediate, and of high virulence were injected intraperitoneally and the subsequent distribution, multiplication, and survival of the bacteria in the tissues and organs of the mouse were studied. The investigation included a comparison of two smooth cultures which differ in virulence but which are indistinguishable culturally, serologically, and in the ability of their 0 antigens to immunize. The most marked difference in the course of infection produced with doses of from 75,000 to 100,000 bacteria per cubic centimeter of these two strains was in the ability of the more virulent to multiply in the mouse after an initial period of lag. The numbers of bacilli in the blood stream and their rate of disappearance from the peritoneal exudates during the first 24 hours were similar with both cultures. No significant difference in resistance to phagocytosis or in toxicity of killed bacilli was demonstrated. An avirulent rough culture was less invasive and less toxic than the smooth culture of low virulence, but showed a greater capacity to survive in the spleen when injected in sublethal doses. While no "virulence" antigen has been demonstrated, virulence did not appear to be conditioned by the presence of the smooth 0 antigen which was closely related to immuniz-

ing capacity. It is suggested that the mechanism of immediate destruction of the bacilli, after intraperitoneal injection, depends chiefly on the mononuclear phagocytes of the omentum and on the reticulo-endothelial system.

M14. The Incidence of Agglutinins for the Paradysentery Bacilli in Normal Human and Animal Sera. H. J. Sears, Marian Schwichtenberg and Lillian Schwichtenberg, University of Oregon Medical School, Portland, Ore.

Of 115 normal human sera tested for agglutinins with antigens prepared from smooth cultures of three different strains of *Shigella paradysenteriae*, Flexner, positive reactions were obtained in 87, 83 and 42 per cent, respectively, at a dilution of 1:20 and in 49, 43 and 13 per cent at a dilution of 1:80. No significant difference was found in the incidence of agglutinins in sera from males and females. Eight specimens of cord blood gave approximately the same results. Of 44 sera tested with one strain of *Shigella dysenteriae*, 10 agglutinated at 1:10. 2 at 1:20 and none at higher dilutions.

Of 121 human sera tested with three strains of *Shigella paradysenteriae*, Sonne, 1.6 per cent agglutinated at least one strain at a dilution of 1:20; none at higher dilutions. The same three strains were agglutinated by 100 per cent of beef, 96.2 per cent of sheep, 93.5 per cent of swine, 46.4 per cent of rabbit and 22.3 per cent of guinea pig sera. Beef, sheep and swine serum titers were generally high; those for rabbit and guinea pig sera low. Absorption experiments indicated that normal agglutinins are qualitatively different from immune agglutinins.

M15. The Influence of Acetyl Group on the Antigenicity of Type I Pneumococcus Polysaccharide. Lloyd D. Felton and Benjamin Prescott, Johns Hopkins University, Baltimore, Md.

The presence of the acetyl group on Type I pneumococcus polysaccharide has been postulated by Avery and Goebel, Enders, Heidelberger, and others, as a necessary characteristic for the antigenicity of this substance. Our work has not confirmed these findings. The present study is taken up from three different angles: (a) treatment of the polysaccharide prepared by the calcium phosphate method (three different samples) with sodium hydroxide according to the method used by Avery and Goebel, with ammonium hydroxide and then sodium hydroxide, and with sodium hydroxide followed by ammonium hydroxide; (b) attempt at saponification of an acetyl group with para-toluidine

and phenetidine; (c) repeated washing with ether, acetone, and methyl alcohol of an antigenic sample which apparently had an acetyl group. The results of analyses of the samples so treated indicate that there is no correlation between acetyl content or glucose number and antigenicity of Type I pneumococcus polysaccharide.

M16. The Effect of Acetic Acid and of Formaldehyde on Pneumococci.
René J. Dubos, Hospital of the Rockefeller Institute for Medical Research, New York City.

Pneumococci resuspended in sufficient concentrations of acetic acid or of formaldehyde retain their characteristic morphology and their positive reaction to the Gram stain. This preserving effect is due to the inactivation of the autolytic enzymes of the pneumococcal cell. The inactivation of the enzymes is partly reversible. Certain components of the autolytic system recover activity when, after removal of the acetic acid or formaldehyde, the cells are resuspended in a neutral medium. Under these conditions, pneumococci undergo partial autolysis; they do not, however, disintegrate, but appear as Gram-negative cocci.

Encapsulated pneumococci, killed with acetic acid or formaldehyde, and in which the autolytic system has been subsequently destroyed or maintained inactive, function as very effective type-specific antigen in the rabbit. On the contrary, encapsulated pneumococci, killed by the same technique, but which have become Gram-negative following reactivation of the autolytic system, fail to incite the production of the type specific carbohydrate antibodies in rabbits immunized by the intravenous route.

These observations emphasize the close relation between the Gram-positive structure of encapsulated pneumococci and the so-called "capsular polysaccharide antigen" of the cell. The results can be used as a basis for the preparation of bacterial suspensions which are stable, and very effective as type-specific antigens.

M17. A Possible Mechanism of "Lowered Resistance" to Pneumonia.
W. J. Nungester and Roy G. Klepser, Hygienic Laboratory, University of Michigan, Ann Arbor, Mich.

The present study is an attempt to determine the mechanism by which exposure to cold, alcoholic intoxication, or deep ether anesthesia tend to predispose an individual to pneumonia. India ink and mucin mixture placed in the noses of rats is aspirated into the lungs in from 40 to 50 per cent of the animals exposed to cold, alcoholic intoxication, or deep

ether anesthesia. Normal rats or rats under light ether anesthesia aspirated the ink in from 0 to 20 per cent of the animals. Pneumococcus culture and mucin mixtures placed in the noses of rats exposed to various conditions developed pneumonia in 13 per cent of the controls, in 42 per cent of the rats exposed to cold, in 38 per cent of the intoxicated animals, in 39 per cent of those under deep ether anesthesia and in 5 per cent of the rats under light ether anesthesia. By physiological methods it was determined that the epiglottis and vocal folds failed to close the trachea, when a stimulus was applied to the posterior pharyngeal region in 18 per cent of the stimulations in control animals, in 54 per cent of the stimulations in rats exposed to cold, and in 46 per cent in alcoholic rats. Alcoholic intoxication and exposure to cold did not increase the mortality rate in rats injected intraperitoneally with pneumococci.

We conclude that one factor in "lowering of resistance" in pneumonia may involve alteration in the normal functioning of the epiglottis and vocal folds.

M18. Some Effects of Pneumococcus Specific Polysaccharide on Red Blood Cells. Louise Fordham Klein and W. J. Nungester, Hygienic Laboratory, University of Michigan, Ann Arbor, Mich.

This work is an extension of a published finding by the authors indicating that the specific polysaccharide of the pneumococcus increases the sedimentation rate of human and rat red blood cells. Red blood cells treated with Type I polysaccharide, centrifuged, and washed, were rapidly agglutinated when brought in contact with specific immune serum, thus suggesting that the polysaccharide was adsorbed on the surface of the red blood cells.

M19. The Distribution of Pneumococci in the Tissues of Pneumonic Rats and Its Bearing on Serum Therapy. Alice H. Kempf and W. J. Nungester, Hygienic Laboratory, University of Michigan, Ann Arbor, Mich.

An attempt has been made to determine the distribution of pneumococci in the tissues of rats with experimental pneumonia. After an intrabronchial inoculation of pneumococci and mucin, rats were killed at intervals. Various tissues and different parts of the affected lung were ground, and the number of viable organisms estimated by plate count. It was found that the advancing portion of lesion in the pneu-

monic lung was rich in organisms, while the older part of the lesion might contain but few bacteria. Relatively few organisms were found in the blood stream or skeletal muscle. On the other hand, the spleen and liver (representing the reticulo-endothelial system) contained a goodly number of bacteria. Small amounts of specific antiserum, when injected at the time of inoculation, uniformly protected animals which were injected with a number of pneumococci equal to the estimated number present in a pneumonic rat at one day. Suspensions of ground infected lung rich in pneumococci were rendered innocuous by the simultaneous injection of immune serum. In contrast, two hundred times as much serum often failed to protect rats when given one day after the inoculation. The failure of delayed serum therapy in rats is discussed from the point of view of the distribution of bacteria in the infected animal.

M20. Bacteriolysins in Gonococcal Arthritis. CHESTER S. KEEFER, Harvard Medical School and the Thorndike Memorial Laboratory, Boston City Hospital, Boston, Mass.

During a study of gonococcal infections it was found that the blood of patients frequently became bactericidal for the homologous strain of gonococcus. An examination of this phenomenon showed that bacteriolysis was accomplished by the blood serum through the combined action of antibodies and complement. Polymorphonuclear cells were not necessary for lysis of the organisms and there was no correlation between the degree of active phagocytosis and the destruction of the organisms *in vitro.*

To determine whether or not the synovial fluid was bactericidal for the gonococcus, various samples of fluid were examined. It was found that gonococci could be recovered from the synovial fluid in only about 25 or 30 per cent of the cases. In the others, it was sterile. On comparing the bacteriolytic power of the blood and synovial fluid, it was found that, when the synovial fluid was infected, it contained no bactericidal properties; when it was sterile, it had the same bacteriolytic power as the blood or it was somewhat less potent than the blood. The difference in some cases was due to the presence of mucin which interferes with the bactericidal action of the synovial fluid.

It would appear from our observations that one of the factors concerned in sterile effusions into the joints in gonococcal infections is the presence of bacteriolysins in the synovial fluid. That there are other factors is evidenced by the fact that in a few cases the fluid remained sterile in spite of the fact that it was not possible to demonstrate bacteriolysins for the homologous organism.

M21. Gonococcus Meningitis and Some of the Difficulties Encountered in Its Recognition. SARA E. BRANHAM, REGINALD H. MITCHELL AND WILLIAM BRAININ, National Institute of Health and Gallinger Municipal Hospital, Washington, D. C.

A case of uncomplicated meningitis due to the gonococcus is reported. Prontylin treatment was followed by recovery. Careful physical examination and painstaking investigation into the history of the patient gave no clue to the source of the infection.

The identification of the organism was made on the basis of growth characteristics, nature of colonies, fermentation and serological reactions, and alkali solubility.

This is the third strain of the gonococcus from spinal fluid which has been studied by the senior author within the last year and a half. These 3 strains have been compared with 9 others from blood stream, joint, eye, and urethral infections. These have all been indistinguishable from each other.

In differentiating the gonococcus from the meningococcus, cultural features, colony type, fermentation reactions, and alkali solubility are of definite value. Serological methods are less satisfactory. Gonococci are well agglutinated, not only by polyvalent antimeningococcic serum, but often by monovalent "typing" serum, some strains showing a close serological relationship to Type II meningococci. The non-specific nature of serologic reactions, and the lack of time for careful study of cultures in diagnostic laboratories may prevent an accurate diagnosis. Gonococcus meningitis is probably more common than it has usually been thought to be.

M22. Experimental and Clinical Observations upon Chemotherapy in Gonococcal Infections. PERRIN H. LONG AND ELEANOR A. BLISS, Johns Hopkins University Medical School, Baltimore, Md.

During the past year we have been observing the effect of sulfanilamide and its derivatives in the treatment of gonococcal infections. Experimental gonococcal peritonitis and septicemia have been produced, through the agency of mucin, in mice. These experimental infections can be cured with sulfanilamide. Clinical data indicate that sulfanilamide is of definite value in the treatment of gonococcal infections in human beings. We will discuss these results and will point out certain lines of clinical investigation which must be pursued, before a true evaluation of sulfanilamide therapy in gonococcal infections can be reached.

M23. The Bactericidal Effect of Sulfanilamide on the Gonococcus in vitro.
Harold F. Wengatz, Ruth A. Boak, and Charles M. Carpenter, Department of Bacteriology, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

Evidence is accumulating that sulfanilamide is a useful therapeutic agent for the treatment of gonococcal infection, yet little is known of the action of this drug on the gonococcus. Accordingly, the question of a bactericidal effect is being studied *in vitro* on recently isolated strains. The technique is as follows. The drug is added to Douglas' broth cultures, both with and without blood, to yield a final concentration of 0.01 per cent. This percentage of sulfanilamide is employed because reports indicate that 10 mgm. per 100 cc. of blood *in vivo* is adequate for successful therapy. Subcultures are made at varying intervals during an exposure of 100 hours to the drug at 36°C.

Ten recently isolated strains of *Neisseria gonorrhoeae* have been studied in this manner. In each instance a marked decrease in the number of viable organisms has been observed after an exposure of 20 hours. Failure to obtain growth in subcultures, however, was not noted until the gonococci had been subjected to the action of the chemical for at least 50 hours. The thermal death time of the gonococcus at 41.5°C. was shortened by about 50 per cent, when the blood broth cultures in which the organisms were subjected to the fever temperature contained the above concentration of sulfanilamide.

M24. Limiting Factors of Sulfanilamide's Action and the Phenomenon of Potentiation.
Ralph R. Mellon and Lawrance E. Shinn, Institute of Pathology, The Western Pennsylvania Hospital, Pittsburgh, Pa.

When two or more minimal effects acting together produce a result that exceeds materially their additive effect, which at the same time is a critical one, a potentiation may be said to result. In terms of bacteriostasis, a critical effect would be a bactericidal one, or close to it. The principle is being applied to correlations between the *in vitro* and *in vivo* effects of a drug, which are so frequently at variance. For example, it has been found that in *Escherichia coli* infections of the urinary tract bactericidal effects of sulfanilamide are in reality a potentiation of the minimal effect on *Escherichia coli* of the urine itself. When the bacterial growth is diluted in broth to the small inocula necessary for a bacteriostatic test *in vitro*, sulfanilamide has no effect on *Escherichia coli* in urine, but when the dilutions are made in the urine, the drug

produces a bactericidal effect. A similar effect on hemolytic streptococci occurs, when 0.9 per cent sodium chloride solution is used as the diluent. This effect, though ordinarily negligible, is often susceptible of being potentiated by high dilutions of sulfanilamide in human serum, so that a bactericidal effect results. But when a broth capable of supporting the growth of two or three cocci is employed for making the dilutions, a negligible degree of bacteriostasis may result. On the other hand, still unidentified factors,—variability of the serum or broth, possibly—may cause the results to fluctuate considerably. Considered as a whole, these results may have a bearing on the validity of our bacteriostatic criteria.

M25. Experimental Production of Gonococcal Septicemia in Mice. ALFRED COHN, Gonococcal Research Unit, Bureau of Laboratories, Department of Health, New York City.

Experimental gonococcal infection of mice was undertaken with some alteration of the technic of Miller and Miller and Castles. A saline suspension of gonococci was diluted to 10^{-1}, 10^{-2} and 10^{-3} in the decanted fluid of a 5 per cent solution of granular mucin (Wilson Laboratories, Chicago, Ill.), pH 7.3, containing 1 per cent of glucose.

One-cubic centimeter doses of the various gonococcus dilutions were injected intraperitoneally into mice. One cubic centimeter of the 10^{2} dilution, containing about 5 million organisms, was the minimum lethal dose. Soon after injection, the animals became sick, and from 80 to 100 per cent of them died within from 18 to 24 hours. Cultures taken soon after death, from the hearts and peritoneums of the infected mice, were usually positive for gonococci. Gonococci were sometimes found in smears from the peritoneum. A few lymphocytes were also seen.

Of 10 gonococcus strains tested up to the present time, 6 produced a gonococcal septicemia in mice. These strains had been subcultured every 2 days for a period of time ranging from 4 weeks to 4 months. These experiments are being continued.

M26. Yeast-Like Fungi Isolated from Normal Skins. CHARLES C. CROFT AND L. A. BLACK. Department of Bacteriology, University of Maryland, College Park, Md.

The finger tips of 100 normal people were cultured for yeast-like fungi by scraping bits of skin and nail into honey broth (pH 5.0–5.4), which was also used with the last 75 persons as an enrichment medium, and by plating on malt agar. Twenty-nine cultures of yeast-like fungi were

isolated from 22 persons, those whose hands were moist much of the time (kitchen workers, laboratory apparatus-washers, and housewives) showing the greatest incidence.

The yeast-like fungi from normal skins and the 13 cultures obtained from pathogenic conditions were classified on the basis of mycelial radiations and colony morphology on malt agar, mycelium in cornmeal agar, detection of ascospores from growth on carrot slants, fermentation of 10 carbohydrates, type of growth and cellular morphology from 1 per cent glucose broth, coagulation of milk, and liquefaction of gelatin.

Of the strains obtained from pathogenic conditions, 9 were classified as *Monilia albicans*, 1 as an unidentified *Monilia*, 1 as a pink *Cryptococcus* and 2 as unidentified yeast-like fungi. From normal skins, 12 cultures either were, or were closely related to, *Monilia parapsilosis*, variations in carbohydrate fermentation and milk coagulation being noted. Three cultures were identified as *Monilia nigra*, 4 as unidentified *Monilia*, 4 as *Endomyces* (species not hitherto reported from normal skin), 2 as *Cryptococcus*, 1 as *Mycoderma*, 1 as *Schizosaccharomyces hominis*, and 3 as unidentified yeast-like fungi. Known pathogenic yeasts were not found on the normal fingers examined.

M27. A Comparative Study of Nineteen Strains of Blastomyces dermatitidis Gilchrist and Stokes, 1898. DONALD S. MARTIN AND N. F. CONANT, Duke University School of Medicine, Durham, N. C.

Since Gilchrist and Stokes in 1898 described and named the fungus *Blastomyces dermatitidis* from American blastomycosis, 21 other names have been applied to the fungus isolated from this disease. Many of these names have resulted from redescriptions of the fungus isolated from a single case. For example, the fungus isolated from a single case was described as *Glenospora Gammeli*, *Glenospora brevis*, *Acladium Gammeli* and *Trichosporium Gammeli* by different authors.

Nineteen strains of fungi were studied, including 13 cultures previously labeled *Blastomyces dermatitidis*. These were compared with cultures of *Glenospora Gammeli*, *Blastomycoides tulanensis*, *Monosporium tulanense*, *Endomyces capsulatus* and *Endomyces capsulatus* var. *isabellinus*. On blood agar at 37°C. and on Sabouraud's agar at room temperature, no significant differences were found by microscopic examination. All produced a yeast-like stage on blood agar at 37°C. and a mycelial stage on Sabouraud's agar, showing *raquette* hyphae and small lateral conidia and chlamydospores. Ascospores could not be found in any of the strains studied.

Serologic tests with human anti-blastomycotic sera showed that all of these strains are similar in their antigenic structure.

The serologic and morphologic evidence support the contention that these 21 new names should be reduced to synonomy with *Blastomyces dermatitidis* Gilchrist and Stokes, 1898, the only etiologic agent in Gilchrist's disease.

M28. A Study of Monilia albicans with Emphasis on Morphological Types and Chlamydospore Production. LYNFERD J. WICKERHAM AND LEO F. RETTGER, Department of Bacteriology, Yale University, New Haven, Conn.

Thirty-five strains of *Monilia albicans* isolated from the vagina, 8 from the mouth, 4 from feces, and 10 from avian sources were studied by a modification of Dalmau's technique for the morphological examination of fungi. These strains could be divided arbitrarily into four overlapping types and a fifth group composed of a few degenerate *Monilia albicans* strains. The fermentation reactions for all four types were identical, but some cultures in the fifth group showed a diminution in the degree to which the more difficultly fermentable sugars were attacked. Since species names have been given to morphological variants representing strains of *Monilia albicans*, it is hoped that the concept of morphologic variation may eliminate many of the species names which now cause confusion in the genus *Monilia*.

All but 5 cultures of the four types produced chlamydospores within 48 hours on cornmeal agar at room temperature; chlamydospores were present in all at the end of 96 hours. Under the conditions of this experiment, the presence of these cells offers proof of the identity of this species, since no other closely related organisms except species in the *Endomyces* genus produce them. However, the *Endomyces*, which are seldom obtained from human sources, may be separated from the *Monilias*, since the latter do not produce ascospores on cornmeal agar, while the former do. Because chlamydospores are distinctive in appearance, and since they occur early in cultures, it is felt that especial emphasis should be placed on their presence in identifying *Monilia albicans*.

M29. The Isolation of Actinomyces bovis from Tonsils. C. W. EMMONS, National Institute of Health, Washington, D. C.

Two hundred pairs of tonsils were examined for anaerobic *Actinomyces*. The tonsils had been removed for the usual causes; no selection

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of cases was made. In no instance was there any evidence of clinical actinomycosis. In some of the tonsils firm cheesy masses, varying from those barely visible to those 3 mm. in diameter, were found. A part of each such mass was examined microscopically. In 37 per cent of the tonsils masses of this sort were found which contained hyphae resembling those of *Actinomyces bovis* in morphology and staining reaction. Other microörganisms were always present. The remaining portions of the masses which contained *Actinomyces*, suitably diluted, were planted in dextrose veal infusion agar shake cultures. Cultures of an anaerobic *Actinomyces* were secured from 14 per cent of the 200 pairs of tonsils, and pure cultures were isolated from 11 per cent. In 3 cases two types of *Actinomyces* were secured from the same tonsil, so that a total of 25 pure strains was studied. Most of these strains have been kept for more than a year. Of these 25 strains, 60 per cent are like *Actinomyces bovis* in morphology, staining reactions, and anaerobiosis. The rest differ by dichotomous branching. Attempts to secure progressive actinomycosis in guinea pigs by inoculation with these strains have not succeeded, but there is some evidence to suggest that repeated inoculations may produce infection. It is believed that *Actinomyces bovis* exists as a saprophyte in human tonsillar crypts.

M80. Statistical Studies of the Virulence of Vaccine Virus. ROBERT F. PARKER, School of Medicine, Western Reserve University, Cleveland, O.

A method is described for estimating the number of particles of vaccine virus which is required to produce infection in the rabbit skin. It depends on the fact that small particles are distributed in a suspension in random fashion, and the probability of obtaining at least 1 particle, at least 2 particles, etc., in a given sample may be calculated by using formulae devised by Poisson. When these probabilities are plotted against the particle concentration of the suspensions, a characteristic curve is obtained for each case. Experimental curves may then be obtained and compared with the theoretical ones. This has been done, using 3 strains of vaccine virus of differing degrees of "virulence." In each instance the best fit of the experimental curve was obtained with the curve which had been calculated on the assumption that one particle causes infection.

M81. Concentrations of Purified Suspensions of the Virus of Vaccinia. C. A. BEHRENS AND G. H. ECHELBARGER, Purdue University, Lafayette, Ind.

Homogeneous emulsions of vaccine calf lymph were prepared by thorough disintegration with ground pyrex-glass, distilled water being used as the diluent. Dispersion of the virus was permitted by keeping the dilutions at from 5° to 10°C. for from 18 to 24 hours. Homogeneity of the emulsions was completed by centrifugation. Clear viable preparations of the virus were produced by removing approximately 85 per cent of the extraneous matter by precipitation at the iso-electric point with M/100 citric acid, centrifugation and immediate restoration of the pH to 7.2. Concentrations of these preparations were obtained by means of vacuum distillation. Nitrogen was also used for the purpose of disturbing the surface of the fluid.

The vaccinal activities of these concentrates, as well as those of unpurified homogeneous emulsions, were determined by intradermal inoculations of 0.2 ml. of various dilutions into rabbits. The titers of both preparations were approximately the same, 1:1,000,000, in terms of lymph.

M32. A Modified Rivers' Culture Medium for Vaccine Virus. August Holm, George F. Leonard and John F. Anderson, Biological Laboratories, E. R. Squibb & Sons, New Brunswick, N. J.

The usual incubation period for vaccine virus in chick-embryo Tyrode solution, according to Rivers' method, is four days. Although alive, the greater part of the embryonic cells in this medium is in a resting stage without mitosis, and necrotizes fairly rapidly. Vaccine virus multiplies best in living mitotic cells, and it therefore seems probable that the potency of the vaccine would be enhanced, if actual growth of the embryonic cells could be induced.

Other workers have shown that peptone in Tyrode solution supports the growth of certain tissue cells. We have found that a Tyrode-peptone solution, autoclaved in a special manner, will allow mitosis of cells from minced chick embryo tissue to continue; that this medium will support growth of vaccine virus to a titer of 10^{-6} or above in 85 per cent of the cultures; that the high initial titer only gradually decreases at temperatures of −5°C. and −12°C.; and that the antigenicity of a liquid virus culture with 50 per cent glycerine is retained after 22 months storage at −12°C., as tested on rabbits.

M33. The Immunizing Properties of Formalinized Cultures of Rocky Mountain Spotted Fever Rickettsiae Grown in Modified Maitland Media. Ida A. Bengtson, National Institute of Health. Washington, D. C.

Formalinized cultures of Rocky Mountain spotted fever *Rickettsia* grown in modified Maitland media prepared from guinea pig serum, guinea pig tunica vaginalis of the testis and Baker's solution have definite immunizing properties against the disease in guinea pigs. Cultures in the first generation, as well as after transfer through a number of generations, are suitable for the preparation of vaccines. Two subcutaneous inoculations of the vaccine at intervals of a week afforded protection against 1 cc. of infected blood inoculated intraperitoneally two weeks after the last administration of the vaccine. The cultures contain a fair number of Rickettsia, but growth is never as luxuriant as is that of the *Rickettsia* of endemic typhus cultivated in the same medium.

MS4. Japanese B Encephalitis Virus: Its Differentiation from St. Louis Encephalitis Virus and Relationship to Louping-Ill Virus. Leslie T. Webster, Rockefeller Institute for Medical Research, New York City.

Six strains of Japanese summer encephalitis B virus obtained from five independent investigators in Japan have proved alike in their reactions in animal species and in their immunological properties, as far as tested. They are immunologically distinct from St. Louis and louping-ill viruses, but very similar to louping-ill in their effects in animals. Mice inoculated intracerebrally or intranasally generally show paralysis of the extremities as a first sign of disease, carry virus in the blood stream during the early stages of infection, and are relatively susceptible to intraperitoneal or subcutaneous injections. *Macacus rhesus* monkeys, given an intracerebral or intranasal injection of a small quantity of virus, develop an acute, fatal encephalitis characterized by cerebellar incoördination and specific necrosis of the Purkinje cells. Sheep injected intracerebrally or intranasally develop an acute, fatal encephalitis, but appear to be resistant to subcutaneous injection.

Japanese B encephalitis and St. Louis encephalitis, according to present knowledge, are therefore distinct. Japanese B and louping-ill viruses, on the other hand, are very similar to each other and to Australian X disease, as described.

MS5. Studies with Poliomyelitis Virus. II. Immunologic Comparison of a Los Angeles Strain of Virus with the M. V. Strain. John F. Kessel, Fred D. Stimpert and Roy T. Fisk, School of Medicine, University of Southern California and the Los Angeles County Hospital, Los Angeles, Calif.

Strains of poliomyelitis virus, as reported in the literature, differ in the severity of the symptoms they produce in 8 monkeys. A few investigators have recorded antigenic differences on the basis of cross immunity and neutralization tests. The present study is an immunologic comparison of a strain of virus, recovered at Los Angeles in 1935, which produces mild symptoms, a low death rate and a high recovery rate in monkeys, with the M. V. strain which produces severe symptoms and a high death rate.

Of 10 animals recovered from infections with the M. V. virus, 4 demonstrated neutralizing antibody to the M. V. virus and of 4 animals 2 were immune to subsequent inoculation with the same virus, while 41 of 44 animals recovered from the Los Angeles strain were found to possess neutralizing antibody to the Los Angeles virus and 16 of 19 recovered animals were found to be immune to a second inoculation with this virus. Of 10 monkeys recovered from the M. V. virus, only 2 exhibited neutralizing antibody to the Los Angeles virus, while 4 of 7 tested were immune to a second injection with heterologous virus. Of the 44 animals recovered from the Los Angeles virus, 18 possessed neutralizing antibody to the M. V. strain and 7 of 15 animals given a second injection were immune to the heterologous strain.

These results demonstrate that common antigens exist in the two strains, but also that they possess antigenic differences, for they do not produce identical immunologic responses in monkeys.

M86. The Blood Stream in Experimental Poliomyelitis. F. B. GORDON AND EDWIN H. LENNETTE, Department of Bacteriology and Parasitology, University of Chicago, Chicago, Ill.

A search was made for poliomyelitis virus in the blood of monkeys during the incubation period and stage of paralysis of the experimental disease. This was accomplished by means of repeated blood transfusions to normal monkeys which received at the same time intracerebral inoculations of starch solution. The transfusions, from 8 to 30 cc. in amount, were taken from intranasally-inoculated and intracerebrally-inoculated donors. The recipients received total amounts of from 43 to 120 cc. of blood. All donor animals developed poliomyelitis, while none of the recipients became infected, and in none was there evidence of an immunological response when tested by direct inoculation and by serum neutralization.

The blood of 7 monkeys with sectioned olfactory tracts was also investigated for the presence of virus during repeated intranasal inocula-

tions. Three were bled for transfusions into normal recipients, which showed no infection and no immunity. Two others of the 7 received starch solution intracerebrally and 2 received horse serum intraspinally at the time of the intranasal inoculations. None of these 7 developed either infection or immunity.

We interpret these results as due either to an absence of virus in the blood stream under the conditions of experimentation, or to the presence of amounts insufficient to be detected by the methods employed.

M87. Fate of Nasally Instilled Poliomyelitis Virus in Normal and Convalescent Monkeys. ALBERT B. SABIN AND PETER K. OLITSKY, Rockefeller Institute for Medical Research, New York City.

This study deals with (1) what happens locally to nasally-instilled poliomyelitis virus in normal and convalescent monkeys, and (2) the capacity of virus to invade the central nervous system (CNS) of convalescent animals. Monkeys were killed at various intervals after the nasal instillation of an amount of virus which constantly produces paralysis in the normal animals (usually 7th to 9th day) but to which the convalescents were previously proved to be resistant. Control studies showed that normal mucosa contained no antiviral factors and that, when as little as 5 or 10 minimal cerebral infective doses were added to it *in vitro*, virus could be detected by the method employed. In normal monkeys virus was found neither in the mucosa (excised with special care to avoid contamination with the olfactory bulbs) nor in the olfactory bulbs at 4, 24, and 48 hours after nasal instillation; in each of 2 monkeys tested at 72 hours, it was demonstrated in small amount (prolonged incubation period) in the nasal mucosa and in comparatively greater quantity in the olfactory bulbs. None was detected in the mucosa on the 4th day or later (including 4 monkeys completely paralyzed on the 8th day), even though the olfactory bulbs remained highly infective. In convalescent monkeys, which received the same amount of virus intranasally, none was found either in the mucosa or the olfactory bulbs at 4 hours, 1, 2, 3, 4, 5, and 7 days; negative results were also obtained with the remainder of the CNS-tested before and after cataphoresis.

M88. Mode of Action of Zinc Sulfate Spray in Preventing Infection with Nasally Instilled Poliomyelitis Virus. ALBERT B. SABIN AND PETER K. OLITSKY, Rockefeller Institute for Medical Research, New York City.

With a virus so potent that all untreated monkeys receiving it intranasally develop paralysis, only zinc sulfate, of many chemicals tested so far, induces resistance with regularity. Among monkeys sprayed daily for 7 days, 95 per cent (18 of 19) resisted virus given 2 days after the last spraying, 90 per cent (9 of 10) at 1 month, 57 per cent (8 of 14) at 2 months, and none of 4 at 3 months. One thorough spraying on each of 2 days, also a single spray, proved equally effective against virus given 2 days and 1 month later. The majority of monkeys (8 of 10) resisted when sprayed 2 hours *after* virus, but only 1 of 4 at 6 hours. Hydrogen peroxide (3 per cent), which rapidly inactivates the virus *in vitro*, failed to prevent infection in 2 monkeys sprayed with it 2 hours *after* virus. Mixtures of virus with 1 and 4 per cent zinc sulfate incubated *in vitro* and then instilled nasally induced paralysis in each of 4 monkeys. No definite correlation was found between acidity, protein precipitating capacity, or ratio of protein to chemical at which precipitates completely redissolve, and the capacity of different chemicals to protect monkeys for prolonged periods. Zinc sulfate is least acid with a pH of 5.2 and other chemicals even more highly protein precipitating, exert little or no protective action. Whether or not the effectiveness of zinc sulfate is due to formation of an intracellular zinc compound which is slowly dissociable, the resistant state disappearing when the dissociation reaches a certain level, is now being investigated.

M39. The Indication of Lymphogranuloma venereum Virus in the Human Intestine by the Use of Bowel Antigen. MOSES PAULSON, Johns Hopkins Hospital and University, Baltimore, Md.

The present study concerns patients with ulcerative colitis of undetermined etiology, in whom the possible presence of a virus in the colon might be related to the colitis, as suggested by a positive intradermal response to inactivated bubo pus due to the virus of *Lymphogranuloma venereum* (Frei reaction and Frei antigen, respectively). Antigens were prepared from grossly fecal-free bowel discharge and diluted 1:10 with Azochloramid. They were inactivated by heat. Controls were secured from patients with colitis, but with negative Frei reactions. An amount of 0.1 cc. was inoculated intradermally in individuals with and without colitis, both with and without positive Frei reactions. The reaction was regarded as positive, if induration or papule was at least 5 mm. at 9 days.

In all, 24 antigens were tested in 58 patients, totaling 409 tests. Six positive antigens were collected from 12 suspected cases. The reaction

to these 6 satisfactory antigens paralleled positive Frei reactions in 86.5 per cent of instances. Falsely positive responses were slightly in excess of 1 per cent. The 12 control antigens, when tested on individuals without colitis, gave falsely positive reactions in 13.3 per cent of those with positive, and 4.4 per cent of those with negative Frei reactions. When tested on patients with colitis, the percentage of falsely positive reactions was higher.

A positive response to bowel antigen indicates the presence of the virus of *Lymphogranuloma venereum* in the material from which the antigen was made. There is striking evidence suggesting etiologic relationship between virus presence and associated colitis.

M40. Virus Forms Present in Scarlet Fever. JEAN BROADHURST AND GLADYS CAMERON, Teachers College, Columbia University and Washington Square College, New York University, New York City.

Extending to scarlet fever the methods used in our work with measles (reported elsewhere) we have observed bodies, non-bacterial in nature, in the nasal membranes and blood of scarlet fever patients and in tissue cultures inoculated with the blood of such patients. In nasal smears nigrosin stain reveals minute, intranuclear bodies, one or more to a cell. These bodies are much smaller than those seen in measles and are more closely grouped.

In blood smears, the mononuclear cells show some of the characters observable in measles,—for example, bubbly eruptions and explosive projections which release rounded bodies of varying size, are noted, as are also definite crescents.

In tissue culture (fibroblast; buffy coat) the changes observed in the direct examination of blood are greatly exaggerated. The mononuclear corpuscles are highly vacuolated and release many definitely staining bodies which are very variable in size. The cells are literally blown apart, much as in measles, but the processes are less violent, less "ballooning degeneration" occurs and fewer bizarre forms are seen. Crescentic bodies are more marked than in measles.

M41. Bacteriologic Studies of Viruses by a New Method. EDWARD C. ROSENOW, Mayo Foundation, Rochester, Minn.

This study is the result of attempts to explain the relationship between the bacteriology of encephalitis and poliomyelitis and the respective viruses. The method consists of making serial dilutions of inocula in different culture media at steps of 10 up to extremely high dilutions

(10^{11}), and then three additional dilutions, each at steps of 1:1,000, (10^{14}, 10^{17}, 10^{20}). Cultures of various materials, including freshly prepared filtrates, in soft glucose-brain agar and glucose-brain broth often yielded pure cultures of streptococci having neurotropic cataphoretic velocity and virulence in extremely high dilutions, when corresponding cultures in soft glucose-agar and glucose broth, without the addition of brain, remained sterile.

M42. Effect of Roentgen Radiation on Papilloma Virus (Shope). J. T. Syverton, R. A. Harvey, G. P. Berry and S. L. Warren, Department of Bacteriology and Department of Medicine (Radiology), University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

As part of an extensive investigation of the effect of radiation on viruses, the effect of roentgen radiation on papilloma virus (Shope) was studied, *in vivo* and *in vitro.*

Irradiation of the virus *in vivo* was accomplished by exposing actively growing papillomata on the ears of rabbits to roentgen radiation (30 cm. target-skin distance, i.e., papilloma base; 200 kv.; filter 0.5 mm. copper, 1 mm. aluminum). Graded massive and fractional doses, from 300 r to 6000 r, were used. It was found that the amount of radiation necessary to "cure" the virus papillomata was between 3000 r and 3500 r, for both types of dosage. Doses of 3500 r or more (whether massive or fractional) were effective, without exception, in causing the complete disappearance of the tumors and in preventing their reappearance.

For roentgen irradiation of the papilloma virus *in vitro* a Berkefeld "V" filtrate of the supernate of a centrifugalized suspension of tumor tissue was used for exposure to unfiltered doses from 5000 r to 3 million r (5 cm. distance from target to the middle of the suspension; 90 kv.; no filter). The infective activity of each sample of the irradiated material was determined by inoculative transfer to cottontail and domestic rabbits, in which latter host the titer also was determined. It was found that a dose of 3 million r had but slight effect on the infective titer in domestic rabbits, and no appreciable effect on the progress and size of the lesions in the cottontail rabbit. The effects of doses of 10 million r, and even greater amounts, are being studied.

M43. Analysis of a Crude Polyvalent Bacteriophage by Specific Absorption and Plaque Purification. Philip Levine, Beth Israel Hospital, Newark, N. J.

Absorption tests of a crude polyvalent Shiga bacteriophage with

heat-killed bacilli revealed that this bacteriophage contains qualitatively different fractions specific for (1) smooth Shiga strains and a particular strain of Flexner, No. 316, and (2) rough Shiga strains and some, but not all, Sonne strains. A third fraction is present which is non-specific and which can be demonstrated in the tests for residual bacteriophage only with the use of several sensitive Flexner strains other than No. 316 Heating this crude bacteriophage at 65°C. for 2 hours served to separate the 3 fractions. Isolated plaques were obtained from the crude bacteriophage and the bacteriophage was propagated with each of the sensitive strains.

With regard to the organisms represented in the two qualitatively specific fractions of the crude bacteriophage, it was found that two sorts of plaques could be obtained. They could be differentiated on the basis of size. From one sort, the bacteriophage produced a lytic filtrate which in its action and heat-stability corresponded to each of the two specific fractions of the crude bacteriophage. The other variety could be obtained from, and propagated with, any sensitive organism, i.e., smooth or rough Shiga strains, any Flexner or Sonne strain, etc. It produced a heat-labile lytic filtrate which was polyvalent in its action. In contrast to the crude polyvalent bacteriophage, this purified bacteriophage produced plaques of uniform size with any sensitive organism. In absorption tests it failed to yield qualitatively different fractions.

M44. Further Observations on Bacteriophage Action in the Presence of Blood. WARD J. MACNEAL AND MARGARET A. MCRAE, Department of Pathology and Bacteriology, New York Post-Graduate Medical School and Hospital, Columbia University, New York City.

While there is quite definite clinical evidence that bacteriophage injected into the blood stream may favorably influence the course of bacteremia due to such organisms as the staphylococcus and the colon bacillus, experiments *in vitro* have shown that whole blood and blood serum tend to prevent the dissolution of bacteria by bacteriophage and make it quite certain that the actual dissolving of the bacteria cannot occur in the circulating blood. MacNeal, Frisbee and Slavkin have shown that a more active phagocytosis by the endothelial cells of spleen and liver is encouraged by the intravenous injection of bacteriophage in the staphylococcus bacteremia of rabbits. d'Herelle and others have

demonstrated an opsonic effect of bacteriophage *in vitro*, but the possible influence of blood or blood serum upon this opsonic effect has remained uncertain.

In the present experiments we have found that bacteriophage exercises a very pronounced opsonic effect *in vitro*, not only when the bacteria are exposed to it in watery menstruum, but also when mixed with blood serum. In fact, the bacteriophage enzymes and the serum seem to reinforce each other in opsonic effect. This mechanism permits the visible demonstration of an anti-bacterial action of the bacteriophage which takes place in blood. This is evidently of practical importance in ridding the blood of the infection, but nevertheless it should be regarded as only a visible sign of the profound influence which the bacteriophage may exert upon the susceptible bacteria.

M45. Susceptibility to Bacteriophage Induced in Naturally Resistant Strains of Bacteria. George Packer Berry and Leslie A. Sandholzer, Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

With a procedure suggested by Griffith's method for transforming pneumococcal types, changes in susceptibility to 3 bacteriophages (Burnet's pure strains C13, C16 and C36) have been induced *in vivo* and *in vitro* in several strains of the *Escherichia* genus.

The procedure *in vivo* was to inject mice intraperitoneally with a mixture of *heat-killed susceptible* organisms and *living resistant* organisms (possessing different biochemical characteristics). Pure cultures of organisms possessing the identical biochemical characteristics of the living resistant organisms employed were recovered from the mice. Of 286 strains isolated by plating the cultures recovered from mice, the percentage lysed by C13 was 42; by C16, 60 and by C36, 21. Of these the percentage of permanently sensitive strains, i.e., sensitive for 10 serial passages, was about 50 for C13, 37 for C16 and 33 for C36.

In the experiments *in vitro*, a resistant strain was transferred serially (10 transfers) in peptone water containing heat-killed cells of the susceptible strain. A total of 300 passages was made with each of 4 resistant strains. Of these 1200 cultures, the percentage becoming sensitive to C13 was 13; to C16, 10 and to C36, 9. None of these sensitive strains retained the acquired lysability beyond 2 serial transfers in peptone water.

In both the *in vivo* and the *in vitro* experiments, the substitution of a

crude extract containing "specific soluble substance" for the heat-killed susceptible organisms failed to induce sensitivity. In other experiments, to be reported later, it was possible to induce permanent and semi-permanent sensitivity by the use of lithium chloride and calcium chloride.

These results are most easily explained on the assumption that the host-parasite relationship in bacteriophagy is intimately associated with the antigenic structure of the host.

M46. Influence of the Bacterial Host on Bacteriophage Regeneration and Specificity. RALPH P. TITTSLER, LESLIE A. SANDHOLZER AND GEORGE PACKER BERRY, Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The titers of bacteriophages C13 and C16 (Burnet's pure strains) varied from 1×10^{-10} to 0 depending upon the particular strain, or even sub-strain, of the *Escherichia* genus employed in the titration. Initially, some strains were susceptible to both bacteriophages, some to only one, others to neither. The following experiments were designed to determine whether different bacterial hosts could alter these bacteriophages.

The bacteriophages were propagated serially for 5–10 passages in peptone water with 5 readily lysable host strains. The titers of the resultant bacteriophages were determined with each of 20 test strains (all were lysed before serial passage). Adaptation to a new host strain was frequently accompanied by the development of a higher titer for that strain; conversely, a loss of ability to lyse some of the initially-lysable test strains was noted, as was a decrease in titer with other test strains.

When it was attempted to carry bacteriophage C13 with 2 bacterial strains that were lysed to a minimal extent only, the bacteriophage failed to regenerate or regenerated to such a slight extent that no bacteriophage was demonstrable after a few passages.

Bacteriophage titration was made by plaque count in a specially devised semisolid agar (agar, 0.5 per cent; peptone, 2 per cent; and meat extract, 0.3 per cent). The use of this medium is new for this purpose and offers many advantages, particularly greater accuracy, over those of other media.

It appears that the specificity of bacteriophage can be altered, both qualitatively and quantitatively, by the bacterial host.

M47. The Elimination of Sensitivities to Bacteriophage from Cultures of Streptococcus lactis. F. E. Nelson and B. W. Hammer, Kansas State College, Manhattan, Kan., and Iowa State College, Ames, Iowa.

Secondary-growth strains isolated from cultures of *Streptococcus lactis* upon which bacteriophage had acted, in some cases from litmus milk cultures and in other cases from plaques, were found to be the same as their parent strains in morphological and cultural characteristics. The sensitivities of these strains to bacteriophages were determined by adding one drop of a 24-hour litmus milk culture of organism and 0.1 cc. of bacteriophage filtrate to 8 cc. of sterile litmus milk and noting whether or not acid production was significantly hindered by the added filtrate. This method checked well with the agar plate-plaque method and was less laborious.

It was found that the sensitivity of the strain of *Streptococcus lactis* to the bacteriophage used was eliminated by the action of the bacteriophage, but sensitivities to the action of some other strains of bacteriophage frequently remained unaltered. By treating the secondary-growth organisms with strains of bacteriophage to which they were sensitive, further sensitivities to bacteriophage were eliminated. By successive treatments, cultures resistant to the action of all available strains of bacteriophage were obtained. Occasionally a partially desensitized culture would revert in one or more sensitivity characteristics, possibly as a result of destabilization by the action of bacteriophage.

Apparently *Streptococcus lactis* is a species characterized by multisensitivity to bacteriophage. These sensitivities differ qualitatively and are capable of independent elimination.

M48. Differential Growth of the Antigenic Types of Staphylococci in Human Blood. Richard Thompson and Devorah Khorazo, Institute of Ophthalmology and Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York City.

Many workers have attempted to establish criteria for differentiating types of staphylococci potentially pathogenic for human beings. The majority of strains from human lesions are now recognized to differ from "non-pathogenic" strains by certain correlated properties. It occurred to us that the presumption of pathogenicity for man of strains with these properties might be strengthened if there could be shown a correla-

tion of such properties with the ability of the organisms to grow in human blood.

Varying numbers of staphylococci of the different antigenic types were mixed with fresh defibrinated human blood and incubated in slide-cells made by the method of Wright. The numbers of colonies developing in the slide-cells were counted and recorded as the per cent of the number of organisms inoculated.

Type A strains consistently showed a greater ability to grow in the defibrinated human blood than did the others. The growth of 18 of the 40 Type A strains tested was not at all inhibited: 36 strains showed less than 50 per cent inhibition and only 1 strain failed to grow. In contrast, 12 of the 61 non-Type A strains were completely inhibited and 56 showed more than 50 per cent inhibition; only 2 of the non-Type A strains were not at all inhibited. In non-defibrinated human blood, the non-Type A strains showed less growth. In serum they grew better, but not as well as the Type A strains.

M49. The Stability of Biological and Biochemical Properties of Staphylococci. JOHN E. BLAIR, Laboratory Division, Hospital for Joint Diseases, New York City.

Although dissociated strains of staphylococci may show a loss of certain biological and biochemical properties, as compared with the parent strains, it appears that there is a tendency for undissociated cultures to retain their original properties over a considerable period of time. This report concerns the stability of the plasma-coagulase reaction, hemolysis, pigmentation, and fermentation of lactose and mannitol over a period of from one to two and a half years. Cultures were kept at room temperature, on brain-heart-infusion agar (Bacto) slants, sealed with cork stoppers, and transferred at irregular intervals, averaging about six months. The following correlations between original and subsequent tests were found: plasma-coagulase, 94 per cent (102 strains); pigmentation, 98 per cent (102 strains); hemolysis, 91 per cent (102 strains); lactose-fermentation, 96 per cent (79 strains); mannitol-fermentation, 97 per cent (70 strains). The fermentation of mannitol and the coagulase reaction ran parallel in 91 per cent of 70 strains during the period studied. Loss of ability to coagulate plasma occurred slightly more often than loss of ability to ferment mannitol.

M50. Attempts to Assay the Enterotoxic Substance Produced by Staphylococci by Parenteral Injection of Monkeys and Kittens. ELLEN

Davison, G. M. Dack and W. E. Cary, Department of Bacteriology and Parasitology, University of Chicago, Chicago, Ill.

The assay of staphylococcal enterotoxic substance by feeding is not a satisfactory method due to the insusceptibility and variation of laboratory animals. Parenteral injection of the suspected substance offers a more delicate test. In parenteral injections, however, the hemolytic, dermonecrotic and lethal toxins also produced by staphylococci cause symptoms conflicting with those due to the enterotoxic substance. Fortunately, these toxins can be neutralized by an antiserum developed against a non-enterotoxic staphylococcal filtrate.

Serum prepared against a filtrate of a food-poisoning staphylococcus, when mixed with filtrates containing enterotoxic substance and injected intraperitoneally into kittens, prevented vomiting; but when the same mixture was injected intracardially into kittens or intravenously into monkeys, symptoms of food poisoning occurred.

If kitten blood be added to the above mixture of filtrate and antiserum and then injected intraperitoneally into kittens, protection is not assured; 4 of 7 kittens vomited after a short incubation period. Thus, it appears that in the presence of whole blood, administered extra- or intravascularly, the enterotoxic substance is not neutralized by antiserum prepared from such strains. In further confirmation of this observation is the fact that 2 of the 3 monkeys immunized to the enterotoxic filtrate vomited when injected intravenously with the filtrate, although none of them reacted when the filtrate was fed. No protection to the enterotoxic substance occurred in monkeys immunized to the filtrate from a non-enterotoxic strain. They vomited when fed a food-poisoning filtrate, and their sera did not in any case protect against the enterotoxic substance.

M51. Immunological Studies in Chronic Staphylococcal Osteomyelitis.

Katherine E. Hite, Sam W. Banks and G. M. Dack, Department of Bacteriology and Parasitology, University of Chicago, Chicago, Ill.

The tendency for recurrence and the prolonged convalescence which frequently follows surgical treatment of chronic osteomyelitis have led us to investigate some of the host-parasite relationships in this disease. A study has been made of staphylococcus antihemolysin titers on the serum of patients with this disease. These tests have been repeated in the same patients over a period of several months. A marked correlation

of the titer with the clinical condition of the patient has been manifest; the titer being elevated at times of acute exacerbation and reduced during periods of quiescence. Injections of toxoid given to patients in the chronic stages, while causing a rise in antihemolysin, failed to alter the course of the disease. Titers dropped rapidly after the cessation of toxoid injection. Since the possibility existed that the staphylococci responsible for the lesions might be deficient in their antigenic properties, filtrates of cultures were assayed for alpha and beta hemolysin and dermonecrotizing toxin production, and strains were studied culturally. While individual variation occurred, these strains differed in no essential respect from toxigenic and pathogenic ones from other sources. Since staphylococci causing osteomyelitis seem to be fair toxin producing strains, and since the antihemolysin content of the patient's serum has been shown to drop in the chronic stages of the infection and to be elevated by toxoid injections without altering the course of the disease, it would seem possible that some host reaction may be influencing antigen-antibody relationships in this chronic condition.

M52. The Value of Strict Anaerobiosis as a Clinical Laboratory Procedure. E. H. SPAULDING AND WILLIAM GOODE, Department of Bacteriology, Temple University School of Medicine, Philadelphia, Pa.

The frequency with which organisms present in routine hospital specimens have failed to develop after aerobic cultivation suggested to us the advisability of employing an environment of reduced oxygen tension. Since semi-solid media proved to be only moderately satisfactory, a strictly anaerobic procedure was adopted.

The present report contains a study of approximately 500 consecutive routine specimens received from the University Hospital. Each specimen was streaked on two blood agar plates. One plate was incubated aerobically; the other was placed in an anaerobic jar. Strict anaerobiosis was secured by evacuating the jar and by refilling with hydrogen activated by the catalytic power of shredded palladinized asbestos. The method has proved practical for routine purposes. Of the 396 specimens showing growth 83, or 21 per cent, were negative aerobically and positive anaerobically. On the other hand, 17, or 4.3 per cent, were positive aerobically and negative anaerobically. The organism appearing most frequently on both plates was a hemolytic *streptococcus*. This type often developed within 24 hours anaerobically while requiring 48 hours to grow aerobically.

A separate series including a semi-solid medium has been studied. The relationship between the sources of the cultures and the occurrence of the different anaerobic species is discussed.

M53. Experimental Subacute Bacterial Endocarditis. PAUL F. CLARK AND PHILLIP E. SVEC, Department of Bacteriology, University of Wisconsin Medical School Madison, Wis.

Until this year our efforts to produce experimental subacute bacterial endocarditis have coincided with the numerous reported failures rather than with the few announced successes. Recently, however, three methods have given successful results. (1) The production of an Arthus phenomenon in rabbits with horse serum, followed by repeated injections of *Streptococcus mitior* (isolated from human cases of endocarditis) suspended in a mixture of broth and horse serum, produced a typical endocarditis in 2 of 11 animals. (2) Acute dilatation of the heart with rapid injections of homologous whole blood and gum acacia in amounts up to 83 per cent of the total blood volume, followed by repeated injections of the streptococci, gave successful results in 7 of 18 rabbits. (3) Large injections of *Streptococcus mitior* (5–10 cc. of a broth culture) three-times weekly for from three to six weeks, produced typical endocarditis in 5 of 9 normal rabbits.

Whichever the method employed, the large, somewhat friable vegetations were usually on the mitral valve, occasionally on the aortic valve, at times on both valves; frequently metastatic infarcts and abscesses of the spleen and kidney were observed. Focal collections of cells, not entirely typical of the human Aschoff body, were present in the heart muscle.

The second method coincides more closely with the conception of a primary injury as an important element in the pathogenesis of subacute bacterial endocarditis. It appears that successful results were obtained by this method with smaller doses of bacteria, especially if the primary injection was given immediately following the dilatation.

M54. The Effect of Washing on Old Strains of Hemophilus pertussis Organisms. JOHN A. TOOMEY AND WILLIAM S. TAKACS, Department of Pediatrics, Western Reserve University and Division of Contagious Diseases, City Hospital, Cleveland, O.

This note reports the effect of washing on *Hemophilus pertussis* organisms. The cells of old strains were repeatedly washed in saline until the supernate was clear. Guinea pigs weighing 250 grams responded alike to 45 mgm. of wet weighed organisms (M.L.D. dose),

whether the cells were washed or unwashed. The washed and unwashed organisms were injected intravenously into rabbits and their immune sera were tested with both washed and unwashed old organisms by methods previously described. Both types of sera gave about equal agglutinin titers with washed and unwashed organisms, a titer which sometimes reached 1:640, although a larger precipitate formed with the unwashed-organism serum and unwashed cells than with washed. Serum produced by injecting washed organisms into rabbits gave approximately equal agglutinin titers (as high as 1:320) with both washed and unwashed organisms, but here also the precipitate obtained with unwashed material was larger, and the supernate clearer, than with washed organisms.

When washed- and unwashed-organism sera were tested for agglutinins with freshly isolated organisms, there were slight differences, the unwashed-organism serum agglutinating either washed or unwashed organisms to 1:80, the washed-organism serum to 1:40.

It is concluded that washing old *Hemophilus pertussis* organisms makes no difference in the virulence of the organism and but slight difference in its ability to produce agglutinins in rabbits. This slight difference, however, may be caused by environmental factors.

M55. Atypical Typhoid Fever Caused by Atypical Strains of Eberthella, MARY A. POSTON, Departments of Bacteriology and Medicine, Duke University School of Medicine, Durham, N. C.

Three patients have been observed in Duke Hospital whose clinical courses have resembled typhoid fever. Atypical strains of *Eberthella* genus were isolated from the blood streams of these patients. The organisms were never recovered from other sources, although 2 of the patients had hemorrhage from the bowel and the third was explored for suspected perforation. The Widal reactions were negative with the "Rawlings" strain of *Eberthella typhosa,* although the patients developed agglutinins to their own organisms in a high titer.

The first strain (Barbour), when freshly isolated from the blood stream, fermented sucrose, produced indol, and was agglutinated by immune typhoid serum only to a dilution of 1:20. After subsequent transplants on artificial media, the organism lost the power to ferment sucrose and produce indol, but it was agglutinated by immune typhoid serum to a high titer.

The second strain (Stanton) gave typical carbohydrate reactions, but it was not motile, nor was it agglutinated by immune typhoid or dysen-

tery serum. However, it was agglutinated by serum from the first case (Barbour). After a number of transplants on artificial media, the organism became motile and it was agglutinated by immune typhoid serum to a high titer.

The third strain (Woodard) gave the typical cultural reactions of *Eberthella typhosa*, but it was not agglutinated by immune typhoid serum, although the strain was agglutinated by the sera from both of the other patients.

M56. Bacillary Dysentery in the Monkey. WILLIAM S. PRESTON AND PAUL F. CLARK, University of Wisconsin, Madison, Wis.

Twenty-seven cases of spontaneous dysentery occurring among *Macacus rhesus* monkeys kept for experimental purposes were studied. The disease was found to resemble closely human bacillary desentery in its clinical and pathological aspects. Seventeen strains of non-motile, Gram-negative, non-sporulating rods were isolated at autopsy from the monkeys, and were found to be identical by biochemical and serological tests, with typical strains of *Shigella paradysenteriae* (usually the Flexner variety) of human origin. Agglutinins were demonstrated in infected animals at titers significantly higher than in a series of normal controls. Three of 4 monkeys fed broth cultures of the isolated strains developed symptoms of dysentery; 2 of the animals died and showed typical lesions post-mortem.

During the course of these studies, 16 strains of motile, Gram-negative, non-lactose-fermenting, non-sporulating rods which ferment glucose without the formation of gas were isolated, in most instances from animals suffering from dysentery. These strains represent previously undescribed organisms which resemble members of the genus *Eberthella*, and which appear to have no causal relationship with the monkey disease, but are of interest as new species.

M57. Concentration of Tubercle Bacilli from Sputum by Chemical Flocculation Methods. JOHN H. HANKS, HAROLD F. CLARK AND HARRY A. FELDMAN, School of Medicine, The George Washington University and the U. S. Public Health Service, Washington, D. C.

Alum flocculations will demonstrate many tubercle bacilli in sputum digests. A method for collecting the bacilli by alum flocculation has been improved and simplified so that it is now proposed as a routine procedure.

The following reagents are required: (a) digestor, 4 per cent sodium hydroxide, containing 0.2 per cent potassium alum and 0.002 per cent bromthymol blue indicator; (b) approximately 2.5 N hydrochloric acid; and (c) 1 per cent ferric chloride in distilled water. The procedure follows: 1. Mix 5 cc. of sputum with an equal volume of digestor, shake and place in a 37°C. bath for 30 minutes. 2. Add the 2.5 N hydrochloric acid drop by drop, with shaking, until the color denotes approximate neutrality. If flocculation does not occur, add 0.2 cc. of the ferric chloride solution and shake again (rarely necessary). 3. Centrifuge the flocculated sample for 5 minutes at top speed and discard the supernate. 4. Smear the precipitate on slides with a loop or capillary pipette, fix by heat, and stain by the Ziehl-Neelsen method.

Quantitative microscopic studies revealed that centrifugation (500 to 1000 times gravity for 1 hour) increased the frequency of organisms per field from 5 to 7 times, while alum flocculation increased the bacilli from 40 to 50 times.

The simplified flocculation method permits (1) reduction of centrifugation time to 5 minutes, (2) filtration through paper as a substitute collection method, (3) preparation of uniform smears, and (4) a greater number of positive diagnoses with less time required for examination.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

A1. Disinfection of Legume Seed by Heat. LEWIS T. LEONARD, Bureau of Plant Industry, U. S. Department of Agriculture, Washington, D. C.

The satisfactory sterilization of the seed of legumes is often unattainable because of an intimate association with microörganisms. While sterilization is desirable, it is not a necessity and the main objective, the elimination of *Rhizobium*, may be accomplished rather easily by heating, since seed will retain germinating ability at temperatures considerably beyond those which are lethal for nodule bacteria. Exposure at 70°C. for 20 hours seems desirable for common disinfection. The presence of a few heat-tolerant organisms is not detrimental, unless these happen to be pathogenic. Open jar or pot cultures, prepared from sterilized material and placed in the ordinary greenhouse, may quickly take on a flora of microörganisms.

A2. Bacteria Using Indol in a Trickling Filter. H. E. CALKINS, R. H. WEAVER AND M. SCHERAGO, Department of Bacteriology, University of Kentucky, Lexington, Ky.

A modification of Winogradsky's silica gel-plate method, as adapted by Weaver, Raidt, and Kerr to the study of trickling filter organisms, has been used for the isolation of indol-utilizing microörganisms from the effluent of the trickling filter at the sewage disposal plant of Lexington, Kentucky. Of 14 strains so isolated, 11 are cocci, which appear to be closely related to *Micrococcus piltonensis* Gray and Thornton and *Micrococcus sphaeroides* Gray and Thornton. In addition, 2 of the remaining 3 strains resemble the phenol-utilizing organisms placed by Gray and Thornton in the genus *Pseudomonas.* It is suggested that there must exist a definite physiological group of bacteria that is intimately connected with the destruction of phenols and indophenols. The first step in the decomposition of the indol by the main group of organisms, the cocci, appears to be an oxidation to indoxyl or to indigotin.

A3. Motility of Protaminobacter rubrum den Dooren de Jong. R. H. Weaver, T. C. Samuels and M. Scherago, Department of Bacteriology, University of Kentucky, Lexington, Ky.

A culture, isolated from an ethylamine silica gel plate which had been inoculated with effluent from the trickling filter of a sewage disposal plant proved to have characteristics identical with those of *Protaminobacter rubrum* den Dooren de Jong, except that the organisms were actively motile with a single polar flagellum. This motility was not observed when the culture was first isolated, but was evident upon later study. A transplant of the culture from which the species was described by den Dooren de Jong, obtained from the Lister Institute, proved likewise to be motile. It is recommended that the descriptions of *Protaminobacter rubrum* den Dooren de Jong and of the genus *Protaminobacter* Nijgh and Van Ditmore, as included in Bergey's Manual, be changed in accordance with this finding.

A4. Motile Colonies of Bacillus alvei and Other Bacteria. Nathan R. Smith and Francis E. Clark, Bureau of Plant Industry, U. S. Department of Agriculture, Washington, D. C.

Colonies of *Bacillus alvei* varying in size from the microscopic, which contain only a few cells, to the macroscopic, which contain hundreds, are motile on the dried surface of nutrient agar plates. On freshly poured plates there is sufficient moisture so that the individual cells spread thinly and colonies are formed only as the plate dries. In the motile colonies the cells have large peritrichic flagella and lie side by side. As

the colony moves forward, débris or other cells are pushed to one side and left behind. Old weak cells of the colony are also continually dropped, leaving a fairly well defined path.

Cultures of bacilli closely related to, if not identical with, *Bacillus alvei* and *Bacillus circulans* have been isolated easily from soil. A small yellow Gram-negative spore-free culture, which also shows motile colonies, was isolated from the intestine of an angleworm. The type of motility of this culture is like that of *Bacillus circulans*, a right or left rotation with a slow forward motion. On the other hand, in the *Bacillus alvei* type of motility the colony moves forward like a bullet and may take any direction, although in general it moves away from the dense growth of the culture to the fresh medium.

It is concluded that the motility of the colonies is not a characteristic of any one species, but that it is the result of a strong motility of the individual cells combined with certain physical conditions of the medium.

A5. Oil Seal for Cultivation of the Sporulating Anaerobes. Robb S. Spray and Alfred R. Stanley, Medical School, West Virginia University, Morgantown, W. Va.

Anaerobic cultivation, especially of broth cultures, as in fermentation studies, is commonly attained by covering the broth with a vaseline or "Vaspar" (Hall) seal. The use, however, of either of these materials is attended with difficultly both in the inoculation and in the removal of samples for staining or other requirements.

There are now available on the market a variety of commercial oils of widely differing viscosities, which offer possibilities as substitutes for vaseline or similar solid seals. We have run serial inoculations in broth protected by such oils. As measured both by the growth of representative species of sporulating anaerobes and by the use of methylene blue as an indicator of anaerobiosis, these tests indicate that broth media may be protected for at least 14 days from the time of autoclaving. Boiling of the culture tubes is not required previous to inoculation.

With the use of an oil of medium-high viscosity inoculations and samplings may be made by capillary pipette directly through the oil without heating the tube, as is required with vaseline or other solid seals. Anyone studying anaerobes under conditions requiring multiple inoculations of numbers of cultures will quickly appreciate this convenience.

A6. Microbiology of the Upper Air. III. An Improved Apparatus and Technique for Upper Air Investigations. BERNARD E. PROCTOR AND BASIL W. PARKER, Massachusetts Institute of Technology, Cambridge, Mass.

An improved apparatus and technique have been developed for the collection, photographic recording and cultural studies of the microflora and particulate matter in the upper air. The apparatus can be sterilized as a complete unit, thereby minimizing contamination during installation or removal from the airship. Moreover, the possibility of contamination in the handling of the samples in the laboratory has been reduced by simplifying the manipulatory technique required.

The collector is an improvement on an earlier model. It has been modified to permit microscopic examination of the material collected, photographic recording and culture with a minimum of manipulation.

Using this apparatus, 40 flights have been made over Greater Boston from November, 1936, through June, 1937. Of the organisms obtained, 16 are bacteria and 19 are molds. The bacteria are predominantly spore formers of the genus *Bacillus*; the molds belong largely in the genera *Penicillium* and *Aspergillus*. Various unidentifiable particles have been recorded on 35 mm. film for further study.

A7. Bacteriological Investigations of the Patuxent River and Chesapeake Bay. KATHERINE CUNNINGHAM AND L. A. BLACK, Department of Bacteriology, University of Maryland, College Park, Md.

The Patuxent River-Chesapeake Bay area surrounding Solomon's Island, is a new and fertile field for bacteriological research. The present study included the isolation and identification of bacteria and observations of the hourly and seasonal variations of the numbers and types of bacteria in the water and mud at fixed sampling stations.

The predominating types of bacteria isolated belonged to the following genera, *Bacillus, Achromobacter, Thiobacillus, Micrococcus* and *Flavobacterium* (members of the last two genera were present in small numbers).

From November through April, the bacterial content of the bottom water was higher than that of the surface water. An increase in the counts of the aerobic bacteria of the surface water began in June, along with a decrease in the counts of the aerobic bacteria of the bottom water. An increase, from June through August, in the anaerobic bac-

terial content of the water at depths of from 15 to 40 m. was correlated with the decrease of dissolved oxygen at these depths. The bacterial content of the surface of the sea floor and the mud to a depth of 9 cm. showed only slight seasonal variation, regardless of the plankton cycles.

A8. The Agglutination Test for Detecting Salmonella pullorum Infection in Chicks. ROGER D. REID, University of Idaho, Moscow, Idaho.

A total of 520 chicks were infected with virulent cultures of *Salmonella pullorum* at 1, 5 and 12 days of age. The mortality was approximately 30 per cent during the first 3 weeks of life. It was possible to recover the organism from the heart's blood, liver and yolk sac in 90 per cent of the chicks that died during this period. Characteristic lesions were frequently observed; most commonly the yolk sac was unabsorbed and the liver showed reddish-yellow areas.

Agglutination tests were performed on the blood of these chicks by the Rapid Whole Blood method and by the Macroscopic Tube Agglutination method at intervals of 2 weeks, beginning when the chicks were a month old. Reactors to the "whole blood" test were found on the first test. At 6 weeks of age 38 per cent of the surviving chicks were found to react by one or both methods. Three per cent more reactors were found by the "macroscopic tube" method than by the "whole blood" method. Reactors were autopsied and *Salmonella pullorum* was isolated from 14 per cent. No further reactors were found after the 14th week.

The results indicate that the "whole blood" method may, under proper conditions, be relied upon to detect most, if not all, of the carriers of *Salmonella pullorum,* and may be valuable in a "control" program. It is further indicated that reactors can be detected before the birds reach "production" age.

A9. A Microscopic Method for the Differentiation of Living and Dead Bacteria in Milk. GEORGES KNAYSI AND MARK B. FORD, Department of Dairy Industry, Cornell University, Ithaca, N. Y.

One cc. of milk is measured into each of two test tubes, A and B. To each is added 0.5 cc. of a dye mixture containing equal parts of 0.2 per cent methylene blue and 0.2 per cent Nile blue sulfate, and the contents mixed. Thus, the final concentration of each dye is 0.025 per cent. In such concentrations, the dyes are not toxic for the milk bacteria tested.

To one tube, say tube B, add a drop of 0.7 to 0.8 N sodium hydroxide and mix. Tube A receives nothing. To each tube add 0.5 cc. of the

melted and tempered hardening agent, which is an agar-gelatin mixture containing 2.0 per cent agar and 8.0 per cent gelatin. Mix the contents of each tube, after the tubes are warmed to about 45°C. to prevent hardening upon mixing.

With a Breed pipette place 0.01 cc. from tube A on the middle of the right half of a clean glass slide. Immediately cover the droplet thus formed with a clean, square cover-glass, 1.28 x 1.28 cm. The droplet forms a film filling the space between the cover-glass and the slide.

Do the same with tube B, placing the 0.01 cc. on the middle of the left half of the slide. Count the bacteria in each preparation and calculate the bacterial content per cubic centimeter. The difference between the count of the preparation from tube B and that from tube A equals the number of viable cells in the milk.

A10. Influence of Time and Temperature of Incubation on Heat Resistance of Escherichia coli. P. R. Elliker and W. C. Frazier, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wis.

To determine the influence of the temperature and time of incubation on heat resistance of *Escherichia coli* (strain H-52), cultures of this organism were grown in sterile reconstituted skim milk for varying periods at 28°, 30°, 30.5°, 38.5°, 40° and 42°C.

At the end of their respective incubation periods, subcultures were made into tubes or flasks of sterile skim milk which were then heated at 53°C. for 30 minutes. Plate counts were made before and after the exposure to heat and the percentage survival of cells during heat treatment was used to compare the heat resistance of the respective cultures.

A decided increase in heat resistance was exhibited by the cultures of *Escherichia coli* during the initial stationary phase of growth. This increase was more marked in cultures incubated at 28°C. than in those incubated at 38.5°C. The time and temperature of incubation of the culture used for inoculum decidedly influenced the degree of the increase in heat resistance during the initial stationary phase of growth of the subculture.

The heat resistance of all of the cultures decreased when reproduction commenced and their resistance fell to a minimum during the period of most active reproduction. The percentage survival then increased to a second peak as the rate of reproduction decreased and the culture entered the maximum stationary phase of growth.

Cultures grown at 38.5°, 40° and 42°C. demonstrated far greater heat

resistance during the maximum stationary phase of growth than did those incubated at temperatures of 28°, 30° and 30.5°C.

A11. The Effect of Subminimal Temperatures upon Streptococcus lactis. OTTO RAHN AND F. M. BIGWOOD, Department of Dairy Industry, Cornell University, Ithaca, N. Y.

Milk cultures of *Streptococcus lactis* were placed at from 0° to 2°C. as soon as the number of cells had reached the maximum. In part of these cultures, the acid was neutralized. Some cultures of each set were kept in an atmosphere of nitrogen.

The number of viable cells remained almost constant for a week or two, and then decreased more rapidly. Neutralization of the acid previous to cooling had only a slight effect on survival, but oxygen was quite important. After cold storage for 3 months, the cultures in air had only a few thousand viable cells per cubic centimeter, while those in nitrogen had over 10 million per cubic centimeter. At this stage, the vitality was low, and the formation of colonies on agar at 30°C. took a long time.

The enzyme content of the cells was measured by the amount of lactic acid formed per cell per hour, by warming the neutralized cultures to 30°C. The fermenting capacity decreased slowly as the time of cold storage increased. In cultures with less than 10 million cells per cubic centimeter, the cells multiplied before sufficient acid had been formed to compute the fermenting capacity. These young cells had a much higher enzyme content.

The chilled cells remained in the water phase of an oil-water surface which indicates that the electro-kinetic surface potential had not been decidedly changed.

A12. Streptococcus salivarius, Streptococcus bovis and the "Bargen Streptococcus." J. M. SHERMAN, PAULINE STARK AND C. E. SAFFORD, Department of Dairy Industry, Cornell University, Ithaca, N. Y.

Streptococcus salivarius, the most common streptococcus in the normal human throat, and *Streptococcus bovis*, the predominating type in the throat and intestines of bovines, show points of kinship, and the validity of *Streptococcus bovis* as an independent "species" has been questioned. A few additional points have been obtained which add weight to the differentiation which has been made between these organisms. Two "100 per cent" differential tests appear to exist in the greater thermal resist-

ance and the greater bile tolerance of *Streptococcus bovis*. Also, there are three strong "majority" differential tests based upon the higher maximum temperature of growth of *Streptococcus bovis* and its usual ability to hydrolyze starch and ferment arabinose, substances which appear never to be attacked by *Streptococcus salivarius*. These tests are supported by several other slight differences.

The identity of the "Bargen streptococcus," believed by some to have a causative rôle in ulcerative colitis, is not clear, though it has been related to the "enterococcus." Our study of a number of strains of the "Bargen streptococcus" indicates that it is not closely related to the enterococci, but belongs to the *Streptococcus bovis* group. Some strains of the "Bargen streptococcus" are identical with typical cultures of *Streptococcus bovis*, so far as studied; other strains agree with the non-starch-hydrolyzing variety of *Streptococcus bovis*. What appears to be the "Bargen streptococcus" has been isolated from the feces of normal individuals.

A13. Prevalence and Classification of Hemolytic Streptococci in Pasteurized Milk. LAWRENCE W. SLANETZ, New Hampshire Agricultural Experiment Station, Durham, N. H.

Weakly hemolytic streptococci were found to be widely prevalent in pasteurized milk. Samples with low standard plate counts sometimes contained from 15,000 to 50,000 of these streptococci per cc. Sheep-blood agar was used for their detection and isolation. Glucose beef-infusion agar proved most satisfactory for maintaining stock cultures. Moderate growth was obtained on tryptone-glucose-skim milk agar, but no appreciable growth occurred on standard beef-extract agar.

The majority of these streptococci were found to enter the milk from utensils which were not cleaned and sterilized thoroughly. Fifty-one strains isolated from samples of pasteurized milk, 7 from utensils and 2 from the teats of cows were studied in detail. These organisms survived in milk when heated to 65°C. for 30 minutes. On sheep-blood agar the colonies appear as the weakly hemolytic or alpha prime type.

The 60 strains were classified into 5 groups, chiefly on the basis of their biochemical and serological characters, as follows: Group I, 12 strains, *Streptococcus bovis*, Var. A, Ayers and Mudge; Group II, 25 strains, *Streptococcus bovis*, Var. B; Ayers and Mudge; Group III, 11 strains, *Streptococcus bovis*, Var. C; Group IV, 8 strains, *Streptococcus bovis*, Var. D; and Group V, 4 strains, *Streptococcus fecalis*. The streptococci classified in Groups III and IV apparently have not been

described previously in the literature. However, it appears advisable at this time to consider them varieties of *Streptococcus bovis*, rather than to suggest new species names.

A14. Characteristics of Staphylococci Associated with Bovine Mastitis.
W. N. Plastridge, F. J. Weirether and L. F. Williams, Storrs Agricultural Experiment Station, Storrs, Conn.

An attempt was made to correlate certain properties of udder staphylococci with other laboratory evidence of mastitis. Quarter samples, collected periodically from animals in four experimental herds, were examined for streptococci, coliform organisms and staphylococci, and as to macroscopic appearance, reaction to bromthymol blue and leucocyte content. The staphylococci were tested for ability to hemolyse cow's blood and to coagulate blood plasma, and for deportment on Chapman's differential media.

Ability to coagulate human blood plasma was found to correlate more closely with accepted laboratory evidence of mastitis than any other staphylococcus test employed. Over 90 per cent of the coagulase-positive cultures were from animals yielding samples containing 500,000 or more leucocytes per cubic centimeter. Of 207 coagulase-positive cultures, 87 per cent hemolysed cow's blood, 90 per cent grew on alkaline bromthymol blue agar, 98 per cent acidified phenol-red mannitol agar (Difco), and 80 per cent were considered positive on crystal violet agar.

Omitting samples showing mixed infection and those collected during early or late lactation, less than 5 per cent of the coagulase-negative cultures were associated with a high leucocyte count. Of the coagulase-negative cultures, 5 per cent hemolysed cow's blood, 49 per cent grew on alkaline bromthymol blue agar and 60 per cent acidified mannitol agar.

The incidence of infection with coagulase-positive staphylococci at any given time varied from 10 to 35 per cent. Less than half of the infected animals gave milk that reacted positively to the bromthymol blue test, and less than 15 per cent gave milk that was abnormal in appearance.

A15. The Effect of Sulfanilamide upon the Streptococci in the Udder of Mastitis Cows and a Method for Its Estimation in Milk. Henry Bauer and Millard F. Gunderson, Department of Pathology and Bacteriology, College of Medicine, University of Nebraska, Omaha, Nebr.

The value of sulfanilamide in the treatment of bovine mastitis was

studied. The amount of the drug present in the blood and excreted in the milk was determined at hourly intervals after the initial feeding. Fuller's method for Prontylin in blood was modified for the estimations in milk. Curves revealing blood and milk levels were obtained. The streptococcic flora of the treated and untreated cows was followed for a period of 60 days by means of Breed smears made from incubated samples of aseptically drawn milk, blood agar plates, bromthymol blue and Hotis tests.

Conflicting results were obtained. In 1 of the 4 treated cows the streptococci disappeared following treatment; appeared upon discontinuance of the drug and were again absent after treatment was resumed. In the other treated cows a decrease in the number of streptococci following treatment was noted.

A16. Reduction in Heat-Resistance of Milk-Bacteria by Milk-Peroxidase.
Karl J. Demeter, Department of Bacteriology, South German Research Institute for Dairy Industry, Weihenstephan-Munich, Germany.

Demeter has shown that peroxidase in raw milk reduces the heat-resistance of *Bacterium coli* under the conditions of "holding" pasteurization. Observations have been extended to bacteria employed in cheese-making (1) because the milk will be pasteurized more and more, and (2) because the cooking of hard cheeses is analogous to "holding" pasteurization. The bacteria examined were *Streptococcus thermophilus, Thermobacterium helveticum* and *Streptobacterium casei.* Heat-resistance was tested by determining the thermal death time of the cultures in high quality, raw milk (R-milk) and also in the same milk freed from peroxidase by heating to 80°C. (H-milk).

Two freshly isolated strains of *Streptococcus thermophilus* were very resistant, surviving temperatures of 65–67°C. for 40–50 minutes in H-milk, but in R-milk they were killed at these temperatures in 5 minutes. A strain of *Thermobacterium helveticum* from Emmental cheese was extremely resistant. Immediately after isolation the thermal death time at 64–65°C. in R-milk was between 10 and 15 minutes, in H-milk between 30 and 35 minutes. A few weeks later the time-periods had increased to 40–50 minutes in R-milk, and to more than 80 minutes in H-milk. Two strains of *Streptobacterium casei* from calves' stomachs were intermediate in heat-resistance, which did not change later. The thermal death time at 63–64°C. was 25–30 minutes in R-milk, 50–60 minutes in H-milk.

Thus, peroxidase reduced the heat-resistance of the streptococci by

about 90 per cent, of the rod-forms by about 50 per cent. The importance of these findings in cheese-making and in "holding" pasteurization is evident, for peroxidase "assists" in killing the bacteria by heat. In "short time" pasteurization this "assistance" is lacking.

A17. Some Factors Influencing the Rate and Extent of Alcoholic Fermentation. B. S. HENRY, Department of Bacteriology, University of Washington, Seattle, Wash.

A comparison of cell counts, water displacement, weight of carbon dioxide lost and total crop yield of yeast, as criteria of the rate and extent of fermentation, was made. The weight of carbon dioxide proved to be the most practical and consistent method.

Using this procedure, it was found that the nitrogen content of loganberry must, as prepared commercially, is not sufficient to produce optimum growth of the yeast strain used, nor to permit complete fermentation. Total nitrogen of 0.25 gm. per liter was found to be the lowest concentration which would give satisfactory growth and alcohol production when the sugar was maintained in excess.

The effect of varying sugar-alcohol ratios upon the rate and extent of fermentation was determined by maintaining glucose concentrations at 5, 10 and 15 per cent in media containing initial concentrations of 0, 2, 4, 6, 8 and 10 per cent. Using grams of carbon dioxide per hour per liter to express the rate of fermentation, it was found that 4 per cent or more alcohol definitely slowed down this rate. However, 10 per cent concentrations of glucose maintained a higher rate at all concentrations of alcohol than did 5 or 15 per cent sugar. The total alcohol content at the end of the fermentation was only slightly influenced by the initial alcohol content. The 10 per cent glucose flasks showed a higher average total alcohol content than did the other concentrations and a higher average alcohol production.

A18. Variations in the Growth and Chemical Constitution of Yeast Grown in Different Media. W. M. CLARK, Columbia Breweries, Inc., Tacoma, Wash.

A study was made of the effect of 25 amino acids, inorganic salts, and carbohydrates on the growth and chemical constitution of a strain of yeast. A single cell of a Froberg, bottom-fermenting type of *Saccharomyces cerevisiae* was picked, cultured in beer-wort and inoculated into Laurent's medium to which were added definite amounts of the test substances. Transplants were made at 24-hour intervals for 128 days. The growth was measured by direct counts.

The number of cells per cu. mm. in each of the 25 cultures decreased sharply and reached a minimum, generally, after 20 transplants. The number then rose gradually until the 40th day after which it remained constant for as long as grown. On the 20th and 128th days, inoculations were made into liter amounts of the respective media, and the yeast crops were collected and analyzed. Chemical analysis of these media at the end of growth showed that, on the average, the yeasts of the 20-day inoculation consumed approximately 14 per cent more carbohydrate and 52 per cent more nitrogen, with a corresponding increase in alcohol production of 6 per cent and a 12 per cent greater pH change, than those of the 128-day inoculation.

After 20 days, all cultures grew well in agar and gelatin stabs and some produced giant colonies on gelatin plates, but after 128 days, no giant colonies could be obtained, and growth occurred only on the surface of the stabs.

A19. Contaminants of Compressed Baker's Yeast. D. R. Colingsworth and I. L. Baldwin, University of Wisconsin, Madison, Wis.

Various brands of compressed yeast cakes, placed at room temperature for five or six days, developed numerous small areas of discoloration due to the growth of various yeasts, molds, bacteria, and actinomycetes. Frequently, numerous black areas of about 1 mm. diameter appeared. The organism associated with the black areas was found to resemble the organisms which are commonly referred to as "black yeasts." On culture media, yeast-like cells of the organism contain a black pigment in the cell wall. A white mycelium is formed under certain conditions. Destruction of sugar does not result in formation of appreciable amounts of acid or gas. On most media the organism grows very slowly.

A medium was developed for the detection of small numbers of this organism in the presence of numerous cells of *Saccharomyces cerevisiae*. A mannitol yeast-infusion agar, to which copper sulfate was added to give a 2 per cent concentration, permitted good growth of the "black yeast," but inhibited the growth of various other organisms, especially *Saccharomyces cerevisiae*. With this medium, three or four "black yeast" organisms per cc. could be detected, although millions of other organisms were present in the sample. Commercial yeast cakes were found to contain from 4 to 100 "black yeast" cells per gram.

A20. d-Lactic Acid Fermentation. C. H. Werkman and A. A. Andersen, Iowa State College, Ames, Iowa.

A new pure culture fermentation process is described for the conversion of glucose into *d*-lactic acid. The organism is an aerobic, sporulating, Gram-positive, motile, catalase-positive, nitrate-reducing bacillus, with an optimal growth-temperature range of from 46° to 50°C.

Good results are obtained with a medium of glucose 15 per cent, soy bean meal 1 per cent, ammonium sulfate 0.15 per cent and calcium carbonate to neutralize. The best results are obtained with aeration. Complete utilization of 15 per cent glucose is obtained within 5 days using a 5 per cent inoculum. Yields of *d*-lactic acid average better than 95 per cent. Soy bean meal may be replaced by malt sprouts, corn meal or skim milk. Acetylmethylcarbinol and 2,3-butyleneglycol are formed in small amounts. A water-white *d*-lactic acid can be prepared directly from the medium.

A21. Nutrient Requirements of Lactobacillus delbrueckii in the Lactic Acid Fermentation of Molasses. H. R. STILES AND L. M. PRUESS, Commercial Solvents Corporation, Terre Haute, Ind.

Good yields and short times of fermentation with *Lactobacillus delbrueckii* depend much on the type and amount of the nitrogenous or accessory nutrients used. These nutrients may be supplied commercially as malt sprouts, steep water, or combinations of either of these with the thin residue of the grain alcohol fermentation. Ammonia nitrogen does not appear as useful a nutrient as organic nitrogen. An abundance of soluble organic nitrogen materially shortens the time of fermentation. Calcium carbonate serves well as a neutralizing agent. Mechanical agitation, and particularly agitation with air, appears to have a hastening effect upon the fermentation.

A22. The Mechanism of Lactic Acid Production by Rhizopus. SELMAN A. WAKSMAN AND J. W. FOSTER, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

Lactic acid was found to be the major product of the metabolism of certain fungi belonging to the genus *Rhizopus*. The formation of this acid is influenced by three groups of factors: (1) biological, comprising the nature and specificity of the organism; (2) nutritional, especially energy source and presence of specific nutrients and stimulants; (3) environmental, notably oxygen tension, temperature and others. The mechanism of the production of lactic acid can be expressed by two phases, the fermentative and the respiratory:

Sugar $\rightarrow$ Lactic acid + Intermediary product (anaerobic)

Intermediary Product + O_2 $\rightarrow$ Lactic acid + CO_2 + H_2O (aerobic)

In the case of these fungi, lactic acid is a final and not an intermediary metabolic product; its accumulation in the culture takes place only in the presence of a neutralizing agent. Zinc stimulates considerably the growth and respiratory coefficient of the organism, but it is not favorable to the accumulation of lactic acid. When a fully grown pellicle is placed in a sugar solution, free from nutrients, active lactic acid production takes place, even in the absence of a neutralizing agent.

A23. A Bacteriological Study of the Bulk Fermentation of Cigar Leaf Tobacco. D. W. McKinstry, D. E. Haley and J. J. Reid, Pennsylvania Agricultural Experiment Station, State College, Pa.

A study has been made of the bulk fermentation of cigar leaf tobacco in the warehouses of a manufacturer. Plate counts of fungi and bacteria were made daily and isolations of predominant types were classified and studied. The fermentation of more than one hundred tons of tobacco, representing the products of all the important cigar leaf districts in the United States, was included in this investigation.

Plate counts of tobacco, made previous to the fermentation, revealed from 2000 to 10,000 fungi per gram. Viable fungi were rarely found following the bulking of the tobacco. Bacterial plate counts, made previous to the fermentation, ranged from values of 50,000 to 250,000. Following the water treatment of the tobacco the counts ranged from 3,000,000 to 5,000,000 per gram. The plate counts exceeded 1,000,000,000 shortly after the bulking of the tobacco. Direct counts increased from 500,000,000, previous to the water treatment, to counts in excess of 10,000,000,000 per gram during the final stages of the fermentation.

Variants of *Bacillus subtilis* were found to be the predominant organisms in a satisfactory fermentation of the leaf. Other members of this genus and cocci were found in lesser numbers.

A24. A Study of the Bacterial Population of Grains Used in a Distillery. A. L. Sotier, Ralph I. Claassen and C. S. Boruff, Hiram Walker and Sons, Inc., Peoria, Ill.

The control of the bacterial population in grain mashes rests, in large part, in keeping out of the mash those grains which show abnormally high bacterial counts. A method for estimating the bacterial population has been developed which is applicable to grains of all sorts, husks of grains, flour, and related materials. The method is accurate, fast, and simple. These studies have resulted in the establishment of bacteriological standards for grains used in a distillery.

A25. *Staphylococcus Food Poisoning from Canned Oysters.* PAUL MAJORS, M. SCHERAGO, AND R. H. WEAVER, Department of Bacteriology, University of Kentucky, Lexington, Ky.

A pure culture of *Staphylococcus aureus,* isolated from the liquor from a can of oysters, the contents of which were incriminated in an outbreak of food poisoning involving two persons (man and wife), produced an enterotoxin when grown and tested on kittens according to Dolman's technique. This is apparently the first instance of staphylococcus food poisoning to be traced to canned food or to oysters.

A26. *The Influence of Diet on the Distribution of Bacteria in the Stomach, Small Intestine and Cecum.* J. R. PORTER, L. WEINSTEIN AND L. F. RETTGER, Department of Bacteriology, Yale University, New Haven, Conn.

This investigation was undertaken to ascertain the bacterial flora, aerobic and anaerobic, in the stomach, certain segments of the small intestine (duodenum, jejunum, upper and lower ileum) and cecum of the white rat. Albino rats were operated upon and the various segments of the alimentary tract clamped-off with hemostats. Standard amounts of the contents from these segments were emulsified in dilution blanks and plated in five media. Incubation of plates was both aerobic and anaerobic.

On a stock diet (calf meal), the stomach and small intestine of the rats were found to contain a significant number of bacteria. Lactobacilli appeared to be normally present in the intestinal tract, being distributed in large numbers from the stomach throughout the small intestine and the cecum. They probably occurred in the smallest numbers in the duodenum. Coli-aerogenes forms rarely appeared in the stomach, duodenum or jejunum; but the lower ileum and the cecum were rich in this type of flora. Sporulating anaerobes were seldom found in sections other than the ileum and the cecum. Very few streptococci and other forms of bacteria appeared in the segments examined. Yeasts were present in all of the sections. The "normal" bacterial flora was altered by certain types of diet. For example, high protein diets brought about an intestinal flora which was free from aciduric organisms. An aciduric flora was again established in the intestinal tract by the feeding of certain carbohydrates.

A27. *Preservation of Grape Juice. V. Pasteurization of Grape and Apple Musts for Storage or Immediate Fermentation.* E. ARTHUR BEAVENS, HARRY E. GORESLINE AND CARL S. PEDER-

SON, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C. and New York State Agricultural Experiment Station, Geneva, N. Y.

The preservation of fresh fruit juices by any heat treatment requires a careful study of the temperatures necessary for pasteurization or sterilization, since the delicate flavors and aromas may be lost and certain chemical constituents coagulated by excessive heating. The purpose of this study was to determine the lowest temperatures necessary to prepare fresh musts for immediate fermentation into wines or preparatory to storage as the unfermented product. In preparing musts for fermentation a majority of the microörganisms has to be killed in order that a pure culture yeast inoculum may predominate; whereas in preparing musts for storage as unfermented juices, it is necessary to kill all microörganisms capable of causing spoilage or fermentation.

Freshly pressed must from Delaware, Elvira, Catawba, and Concord grapes and the Baldwin apple was heated in several types of pasteurizing equipment including the open kettle, the continuous flow ribbon and electropure pasteurizers. Plate counts from samples of musts heated in the three types of pasteurizers at 60°C. (140°F.) showed marked reductions in the number of microörganisms. All microörganisms were killed when heated in the open kettle at 73.9°C. (165°F.), while complete sterilization was not obtained in the other two types. Heating of Baldwin must in the three types of pasteurizers at a temperature of 65.5°C. (150°F.) caused a marked reduction in the number of microorganisms.

There were only slight differences in the chemical compositions of wines made from the various pasteurized musts, and organoleptic tests of these wines failed to give any indication of burned or "off" flavors.

A28. Bacteriological, Cytological, Zymological and Chemical Studies of the Cause of Cucumber Pickle Spoilage. F. W. FABIAN AND E. A. JOHNSON, Department of Bacteriology and Hygiene, Michigan State College, East Lansing, Mich.

A strain of *Bacillus mesentericus-fuscus* was isolated from spoiled salted cucumbers. This organism was capable of causing "slips" within 6–12 hours and "mushy" pickles within 12–24 hours. It grew readily in salt concentrations of 9–11 per cent. Its acid tolerance in these concentrations of salt ranged from 0.15–0.20 per cent acetic acid and 0.2–0.3 per cent lactic acid.

Zymological studies demonstrated that all the members of the

mesentericus-megatherium group worked with produced protopectinase, pectase and pectinase, as judged by chemical and cytological studies. The enzymes showed a greater acid but less salt tolerance than the organisms. Slippery pickles were produced in 1 per cent acetic acid and 0.6 per cent lactic acid within 5 days.

Cytological studies of normal pickles and those cured in either salt or dill brines showed the presence of pectic materials in the middle lamella, when stained with ruthenium red, whereas pickles that had become "slippery" or "mushy" showed diminution or complete absence of pectic materials respectively. Pickles treated with ammonium oxalate and acids such as hydrochloric, acetic, and lactic, as well as those which were cooked, were also studied cytologically.

Chemical studies were made of the pectic content of normal and spoiled pickles to check the cytological studies. The results indicated that, when pickles become "mushy" due to bacterial action, the total pectic content is normal. A twofold increase of soluble pectin occurs in spoiled pickles as compared with that in normal. Chemical studies also demonstrated the limited interpretation that may be obtained with ruthenium red.

It was demonstrated that pickle spoilage may be caused by bacterial enzymes which dissolve the pectic materials in the middle lamella or by acid hydrolysis which loosens the protopectin from the cellulose of the cell walls.

A29. A Bacteriological Study of Raw Cane Sugar Plants. Carl S. Pederson, New York State Agricultural Experiment Station, Geneva, N. Y.

The growth of certain bacteria, particularly slime-producing types, has been a source of annoyance and sometimes serious loss in factories producing raw sugars from cane and beets. The microörganisms are introduced with the incoming cane and beets and measures must be taken early in the process to prevent their growth. Studies were conducted to show the possible sources of infection in certain Cuban cane sugar plants, the type of organisms involved, and the methods of elimination.

After thorough examination of the equipment, plate and microscope counts were made from samples of the juice taken from various parts of the equipment. Over 300 cultures were isolated and identified. Cultures of *Leuconostoc mesenteroides* (Cienkowski) Van Tieghem were obtained from a large number of the samples. Various spore formers,

micrococci, Gram-negative rods, as well as yeasts and molds, were also found.

From these studies, it seems obvious that the growth of microörganisms can be controlled in well equipped plants by practicing cleanliness and by the rapid handling of the cane and juice previous to, and during, heating.

A30. Survival of Thermophilic Food-Spoilage Organisms in Stored White Beet Sugar. H. H. HALL, Food Research Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

Periodic examinations of stored white beet sugar showed decreasing numbers of spores of thermophilic food-spoilage organisms. Examination of sugars by the National Canners Association methods showed that the spores of *Bacillus stearothermophilus* Donk are the predominating type and that they are sometimes present in numbers exceeding the limits established for sugar of canning grade.

Storage of sugar for several months after its production often results in the mortality of sufficient spores to improve its quality to that of canning-grade sugar. The influence of containers, original contamination and sugar impurities were considered as being causes for the decreasing numbers of spores during storage.

A31. A Comparative Study of the Use of Varying Concentrations of Agar in the Test Medium Used to Detect Contaminations in Biologic Products. CAROLYN R. FALK, HELEN BIENIASZ AND MARGARET SIMMONS, Bureau of Laboratories, Department of Health, New York City.

In previous papers on the bactericidal action of antiseptics, and in the routine testing of biologic products for sterility, we have noted that bacterial growth sometimes appears in poured agar plates but not in broth fermentation tubes, and *vice-versa*. These observations suggested that neither a solid nor a liquid medium alone is suitable for the detection of all common contaminating organisms. For this purpose Hitchens and others have recommended the use of 0.1 per cent agar ("Hitchens' medium") in conjunction with broth.

We have tried to determine whether any single yet simple medium could be used with the assurance that all contaminating bacteria would be found. The effect of gradually decreasing the agar in the test medium from 2.0 to 0.0009 part per hundred on the growth of staphy-

lococci, diphtheroids, aerobic spore-bearing bacilli, streptococci, *Escherichia coli* and *Pseudomonas pyocyaneus* was studied, when the test organisms were added to broth, and when they were added to typical biologic products containing preservative. Products known to be contaminated were employed. Incubation was at 37°C., and observations were made daily for a week.

The results show that contaminating bacteria are detected more readily in broth containing small amounts of agar (0.25 to 0.06 part per hundred) than in broth in fermentation tubes, or in poured agar plates, provided either is used alone. Hitchens and Spray have made similar observations. To establish the practicability of these findings we have used routinely 0.1 per cent agar together with the regular "sterility test" broth for eight months, finding it a reliable and simple medium to use.

INDEX OF AUTHORS

THE ENTEROCOCCI AND RELATED STREPTOCOCCI[1]

JAMES M. SHERMAN

Cornell University, Ithaca, New York

Quite out of line with the usual "Presidential Address" I have chosen to speak to you briefly about some of our work of the past few months. These studies have concerned the extension of the Lancefield serological technique to those species or types of streptococci which are usually considered "enterococci," as well as to certain related forms. In doing this I must make use of material which belongs quite as much to others as to me. These investigations have been made possible during a comparatively brief span of time, amid many other duties, only by the untiring industry of my associates, Dr. Floyd R. Smith, now of the University of California, and Mr. Charles F. Niven.

II

"The enterococcus," as this term is commonly used among bacteriologists, has about as much biological meaning as "the bear." The name enterococcus has indeed covered a multitude of sins and has served manifold purposes, not the least being that of a screen behind which the investigator could hide his ignorance of the organisms with which he worked. A few reactions only have been necessary in order to classify an organism as an enterococcus. Such properties as thermal resistance, bile tolerance, reducing action, and the fermentation of mannitol are in general characteristic of enterococci, but by no means peculiar to them. The ability to ferment mannitol has been considered a prime requisite for an enterococcus, but one important species in the group does not attack this substance.

In our own work on streptococci we have applied new criteria

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through which has evolved what we consider to be a true, or at least useful, enterococcus division of the genus *Streptococcus* (Sherman and Stark, 1934; Sherman, Mauer and Stark, 1937; Sherman, Stark and Mauer, 1937; Sherman and Wing, 1937; Sherman, 1937). So defined, the now known types of enterococci are the non-hemolytic *Streptococcus fecalis* and *Streptococcus liquefaciens*, and the hemolytic *Streptococcus zymogenes* and *Streptococcus durans*. Thus far, we have not encountered other streptococci which fully meet our specifications for an enterococcus, although, as will be shown later, at least one type comes close to so doing. However, it is not likely that such a nicely limited and picketed subdivision will long remain unviolated as our knowledge of the streptococci increases.

III

Streptococcus zymogenes was discovered by MacCallum and Hastings (1899), as the causative organism in a case of endocarditis. Although it has been reported from time to time through the years from cases of endocarditis and from human stools, this long-known organism has nevertheless remained little known. For fifteen years, this hemolytic streptococcus masqueraded around the laboratories of the most distinguished medical research institution in the world as a "dairy organism." In the meantime, this same streptococcus had been encountered by workers in dairy laboratories who, identifying it with the organism of MacCallum and Hastings, considered it the private property of the medical bacteriologist. It was upon cultures of this organism, isolated many years before from cheese, that Lancefield (1933) established her group D hemolytic streptococcus. Later, Lancefield and Hare (1935) obtained cultures of group D hemolytic streptococci from the human vagina and related them to the intestinal enterococci. On the basis of cultural studies of a few of Dr. Lancefield's cultures, the group D streptococcus was identified more specifically with *Streptococcus zymogenes* (Sherman, Stark and Mauer, 1937), and it is further shown in a paper now in press (Smith, Niven and Sherman, 1938) that all of the cultures contained in a large collection of *Strepto-*

coccus zymogenes, which had previously been identified by physiological reactions, were found serologically to belong to the Lancefield group D.

Although the Lancefield group D was established on cultures of *Streptococcus zymogenes*, and this is the only streptococcal type which has specifically been identified with that group, it is probable that some of the group D organisms which have been reported have in fact belonged to the hemolytic *Streptococcus durans* type, rather than the prevailing intestinal *Streptococcus zymogenes* which may be considered the most typical form of "hemolytic enterococcus." The unique characteristics which differentiate and widely separate these types from the other hemolytic streptococci have been presented in a number of papers, and need not be reiterated.

IV

An important addition to our knowledge of the serological grouping of streptococci has been made by the finding of Lancefield (1937) that strains of non-hemolytic enterococci, which had been identified as typical cultures of *Streptococcus fecalis* and *Streptococcus liquefaciens* by cultural studies and biochemical tests, belong to her group D. This development was of much interest to us from several points of view, and we have, therefore, sought to extend these observations.

More than fifty cultures of *Streptococcus fecalis*, which had been identified by physiological studies, have been tested serologically. These cultures were isolated from human feces, milk, ice cream and cheese; included also was one strain obtained in pure culture from a fatal case of endocarditis, sent to us by Dr. E. G. D. Murray, and a culture of a motile streptococcus, isolated from milk by Dr. C. B. van Niel. Without exception, these cultures were found to belong to the Lancefield group D. Likewise, more than twenty cultures of *Streptococcus liquefaciens*, isolated from milk and from plants, have been tested, and all gave good reactions with group D serum. It may therefore be said with fair assurance that *Streptococcus fecalis* and *Streptococcus liquefaciens* are both members of serological group D.

V

For more than ten years we have had a special interest in a hemolytic streptococcus, *Streptococcus durans*, which has been isolated from time to time from dairy products and which, innocently enough, has been at times a serious plague to the industry because of the overzealousness of health officials. The basic characteristics of this organism, which clearly mark it as different from any of the disease-producing hemolytic streptococci, were long ago determined and published. Purely on the basis of physiological characteristics, *Streptococcus durans* was related to the enterococci (Sherman and Wing, 1937), and more recently (Smith and Sherman, 1938) it has been found to be in fact an intestinal organism, apparently ranking second only to *Streptococcus zymogenes* among the prevailing hemolytic streptococci found in human feces.

It is true that if one wished to make the same fine distinctions, based upon the fermentation tests, as some recommend in the establishment of species among the hemolytic streptococci belonging to the Lancefield group A, many "species" would have to be recognized among the enterococci; but when minor fermentative differences are ignored, the three well-known enterococcus species, *Streptococcus fecalis*, *Streptococcus liquefaciens* and *Streptococcus zymogenes*, are very closely related. This relationship appears to be so close that we have contended elsewhere (Sherman, Stark and Mauer, 1937) that these types might well be considered as one species with its respective varieties. *Streptococcus durans,* on the other hand, is not so intimately related to the other known enterococci, and is separated by a sufficient number of correlated characteristics to justify, perhaps, a distinct specific designation. In table 1 are given a few selected characteristics which appear to substantiate this view.

Streptococcus durans has also been serologically identified as belonging to the Lancefield group D, but not without some difficulty. At first, when we had succeeded in making only rather poor group D sera, extracts of *Streptococcus durans* gave negative results, though slight reactions were obtained with some strains. Yet these sera were good enough to identify *Streptococcus zymo-*

genes, Streptococcus fecalis and *Streptococcus liquefaciens*. However, when potent group D sera were obtained, prepared against cultures of *Streptococcus zymogenes,* all cultures of *Streptococcus durans* gave positive reactions. In order to settle unequivocally the serological identity of *Streptococcus durans,* we have sought to make a group D antiserum using this organism as the immunizing antigen. All of these attempts resulted in complete failure until very recently, when we did succeed in obtaining an anti-durans group D serum which reacts with the extracts of the other enterococcus species, as well as with those of *Streptococcus durans.* It can now, therefore, be concluded that *Streptococcus durans,* in common with the other three enterococcus types,

TABLE 1

Some selected characteristics showing the interrelationships of the enterococci

SPECIES	HEMOLYSIS IN BLOOD AGAR	GELATIN LIQUEFIED	STRONG REDUCING ACTION	GLYCEROL FERMENTED	MANNITOL FERMENTED	SORBITOL FERMENTED	SUCROSE FERMENTED
S. fecalis......................	−	−	+*	±	+	+	±
S. liquefaciens.................	−	+	+*	+	+	+	+*
S. zymogenes....................	+	±	+*	+	+	+	+*
S. durans.......................	+	−	−	−	−	−	−*

* Occasional variation from type reaction; extremely rare exceptions not noted.

Streptococcus zymogenes, Streptococcus fecalis and *Streptococcus liquefaciens,* does belong in group D.

It has been shown that all of the four now recognized species of the enterococcus group, as this group has been defined by us, belong to the Lancefield group D. More than two hundred cultures representing these four types, which had previously been identified by means of cultural tests, have been serologically identified as group D organisms without a single miss. This looks easy, and it was in fact easy; but it was easy only because we had, so to speak, first caught our rabbits. The cultures had been identified by careful study and the application of a broad series of tests. With none of the previous standards which have been used for the identification of enterococci would such a correlation have been possible.

VI

Streptococcus lactis is a bacterium about which I can speak with much enthusiasm, considerable affection, a bit of pride, and even some heat. This, I feel sure, is the organism which a distinguished governor of a progressive state, preëminent for its dairy industry, had in mind when, upon welcoming the members of this Society to the State Capitol, he addressed us on the powers and magic of "This Bacteria."

My own interest in streptococci has been superficial, largely taxonomic in nature, aimed chiefly at the differentiation and identification of those species, of little general interest, with which we have had to deal in our work. If there is anything of originality which has been applied in these expeditions of mechanical moss gathering, it is contained in the work done many years ago (Sherman and Albus, 1918) which established for the first time the precise identity of *Streptococcus lactis.* The definition then arrived at has stood thus far. But the similarity of *Streptococcus lactis* and *Streptococcus fecalis* in certain characteristics was noted years earlier, and *Streptococcus lactis* was not recognized as an independent species in the better-known early classifications of the streptococci. Although differences between these organisms have been pointed out from time to time in papers from our laboratory, most of the supposedly more authoritative compilations on the streptococci have not admitted *Streptococcus lactis* as a specific type. It is frequently dignified by some such reference as "the so-called *Streptococcus lactis* of the dairy workers." The "dairy workers," on the other hand, have had full confidence in the integrity of their organism as a distinct biological entity, and, as is usually the case, it is those who have worked intimately with the subject who know what they are talking about.

The finding that *Streptococcus fecalis* is a member of the Lancefield group D was indeed grist for our mill. We have naturally had an interest in testing *Streptococcus lactis* extracts against group D antisera. A substantial number of cultures have been so tested and not one has given the slightest reaction. *Streptococcus lactis* is, therefore, as clearly differentiated from *Streptococcus fecalis* serologically as it is physiologically.

In this connection we have made a few attempts, not thorough ones, to produce an anti-lactis group serum using *Streptococcus lactis* as the immunizing antigen. We have been rewarded only with sera which are type-specific but not group-specific. This, perhaps, is the expected finding in view of the works of Hitchcock (1924) and of Lancefield (1925a, b) on the non-hemolytic streptococci. But our few trials do not justify a conclusion that *Streptococcus lactis* contains no group antigen; only that it does not belong to serological group D, of which *Streptococcus fecalis* is a member.

At the risk of boredom and needless repetition, the characters which differentiate *Streptococcus lactis* from *Streptococcus fecalis* are given in table 2.

TABLE 2

Some selected characteristics of Streptococcus fecalis and Streptococcus lactis

SPECIES	LANCEFIELD GROUP D	GROWTH AT 45°C.	GROWTH IN 6.5 PER CENT NaCl	GROWTH AT pH 9.6	SORBITOL FERMENTED	GLYCEROL FERMENTED	MANNITOL FERMENTED
S. fecalis	+	+	+	+	+	±	+
S. lactis	−	−	−	−	−	−	±

This would appear to provide ample grounds upon which to base even a conservative "species" rating among bacteria.

VII

Among the clearly defined types of intestinal streptococci is *Streptococcus bovis*. This organism was first differentiated as a definite species by Orla-Jensen (1919) and its description has been further amplified by a few subsequent investigators (Ayers and Mudge, 1923; Sherman and Stark, 1931). It is the predominating streptococcus in the bovine mouth and intestine, but it also occurs in smaller numbers in the human intestine. *Streptococcus bovis* is more closely related to the so-called viridans streptococci and only rather remotely related to the true enterococci. One would have to have small regard indeed for physi-

ological characteristics, to classify *Streptococcus bovis* in the same group with the enterococcus streptococci. This is shown in table 3.

However, *Streptococcus bovis* does have some characteristics in common with the enterococci, such as the ability to grow at high temperatures, thermal resistance, and bile tolerance. In addition, a substantial proportion of strains of *Streptococcus bovis* ferment mannitol, though the majority do not attack this substance. A number of investigators have classified organisms of the *Streptococcus bovis* type as enterococci.

For the present work we had at our disposal a collection of more than one hundred strains of *Streptococcus bovis* which had

TABLE 3

Some selected characteristics of the enterococci and of Streptococcus bovis

SPECIES	GROWTH AT 10°C.	GROWTH IN 6.5 PER CENT NaCl	GROWTH IN 0.1 PER CENT METHYLENE BLUE	GROWTH AT pH 9.6	NH_3 PRODUCED FROM PEPTONE	STARCH HYDROLYZED	RAFFINOSE FERMENTED	INULIN FERMENTED
Enterococci	+	+	+	+	+	−	±*	−
S. bovis	−	−	−	−	−	+*	+	±*

* Some strains of *S. bovis* do not hydrolyze starch; the majority of the enterococci do not ferment raffinose; the majority of strains of *S. bovis* ferment inulin.

been isolated and carefully studied by Mrs. Pauline Stark. This collection included strains from the bovine mouth, bovine feces, beet pulp, normal human feces, and some additional strains which had been isolated from cases of ulcerative colitis and identified as the "Bargen streptococcus." Extracts of all of these cultures were tested with potent group D antiserum.

About one-half of the *Streptococcus bovis* cultures gave definite reactions with group D serum; about one-third gave absolutely negative results; while the remaining cultures gave slight "plus-minus" tests which, however, could not be confused with a definitely positive reaction. Among the cultures from each of the various sources enumerated above were some which gave posi-

tive reactions and others which were negative with group D serum.

The exact meaning of these reactions between *Streptococcus bovis* and antiserum prepared against group D streptococci would probably best be left for interpretation by a competent immunologist. Even if we were competent to judge, our work is not sufficient to justify an opinion as to whether this is merely a cross reaction or whether the enterococci and *Streptococcus bovis* have in fact a common antigen. We have, however, performed some simple absorption tests which may be worth mentioning. When absoption was attempted with whole cells of *Streptococcus bovis* some technical difficulties, probably not insurmountable, were met, so we resorted to absorption with the unconcentrated extracts of the cells. This involves a dilution of the serum, but it was sufficiently potent to give good reactions after being so diluted. Serum which had been absorbed with *Streptococcus zymogenes* extracts gave no reactions either with *Streptococcus zymogenes* or *Streptococcus bovis*. Although we were not able, within the allowable limits of dilution, to remove completely the antibodies of the serum when extracts of *Streptococcus bovis* were used, the titer appeared to be reduced to about the same degree for each of these organisms, only very faint reactions being obtained.

In order properly to elucidate the serological relationship of *Streptococcus bovis* to the enterococci, it would be desirable to have anti-bovis serum. We have made two attempts to produce anti-bovis group sera without success, though good type sera were obtained. Influenced by the results of earlier work on the non-hemolytic streptococci, we may have discontinued the experiments too soon when the expected result was obtained.

In connection with the results obtained with *Streptococcus bovis*, it is pertinent to mention a few tests which have been made with other viridans streptococci with group D antiserum. The *Streptococcus equinus* of the intestine of the horse shows some points of relationship to *Streptococcus bovis*. A number of cultures of *Streptococcus equinus* have been tested with group D serum but none gave a positive reaction, though half of them

gave very slight precipitates. Cultures of *Streptococcus salivarius* from the human throat gave only negative reactions. *Streptococcus thermophilus*, which has in common with the enterococci high thermal resistance, but which could not be considered otherwise closely related, was also tested with group D serum and all cultures gave completely negative results.

VIII

We have in our possession six cultures of a streptococcus which does not belong to the Lancefield group D, but which would easily qualify as an enterococcus according to any previous definition of that group. It almost meets our own specifications; in fact, it straddles our fence, and is clearly more closely related to the true enterococci than to any other group of known streptococci. The streptococci show no more respect for the barriers which we have constructed than they do for those erected by others; they refuse to remain neatly placed in their respective cages.

The cultures of this streptococcus, isolated some years ago from human feces, caught the ever alert eye of Mrs. Stark, who recognized them as being quite unusual, and preserved them. They resemble *Streptococcus fecalis*, but diverge from it in some characteristics which seemed sufficiently significant so that we did not so classify them when making a study of that species a few years ago.

The experience with this enterococcus-like organism, that with *Streptococcus lactis*, and the perplexing results obtained with *Streptococcus bovis*, make it amply clear that had we started with the usual hazy conception of the nature of enterococci, this serological excursion could have resulted only in utter confusion, so far as the non-hemolytic organisms studied are concerned. But with more rigid physiological criteria, the cultural and serological findings dovetail very nicely indeed. From sources where, so far as published records reveal, an "enterococcus" is very superficially defined, reports have come to the effect that all enterococci belong to one serological group. Unless critical standards are used in the definition of an enterococcus, such

claims can be accurate so far as, and only so far as, habitat and statistical probability operate in their favor. True enough, when the source of the isolations is limited to the human intestine, chance alone might cause a very high percentage of hits; but the conclusion, under the conditions indicated, must remain essentially without substance. They must first catch their rabbits.

IX

In summary, the results of our attempts to extend the Lancefield serological technique, with especial reference to group D, to the enterococci and certain other streptococci, are presented in table 4.

In the study of streptococci a wedge, quite artificial in my opinion, has been driven deeper and deeper between the hemolytic and non-hemolytic species. Even the methods of study long ago began to diverge. Most of those interested in the hemolytic forms have long tended to limit their studies to a few reactions, apparently satisfied that the proper cultural tests had been found. Among the workers on the non-hemolytic streptococci were a few restless souls who kept increasing the number of tests used in the hope of ultimately finding some better ones. It is fair to say that before the advent of the Lancefield technique, more progress had been made in the classification of the non-hemolytic streptococci than in classifying the hemolytic types. Now, of course, the situation is quite reversed. Only time can tell just what sort of rude awakening is in store for us when methods comparable in accuracy to the Lancefield precipitin technique are developed for the non-hemolytic streptococci. From the results of our efforts to correlate the physiological and serological findings with the enterococci, I am encouraged to believe that sound progress has been made in the differentiation of at least some of the non-hemolytic streptococci.

The crying need in bacteriology is for new, not standard, methods. Standardization is ultimately desirable, but it is a vicious mirage during the formative stage of development. Bacterial taxonomy will not be put on a sound basis until better methods are employed; and I know of no way to arrive at new and im-

proved techniques without the employment of many tests. Again, it is necessary, at the present time at least, to make use of many reactions which appear to be without value, since no one can predict which ones will prove useful when applied to new organisms. I have mentioned the case of the enterococcus-like streptococcus which does not belong to the Lancefield group D. This streptococcus differs from *Streptococcus fecalis* in five physiological characteristics. Regardless of what one may choose to consider a "species," a difference in five characters is ample to

TABLE 4

The enterococci and certain non-hemolytic streptococci in relation to the Lancefield group D

SPECIES	DIVISION	GROUP D
S. fecalis	Enterococcus	+
S. liquefaciens	Enterococcus	+
S. zymogenes	Enterococcus	+
S. durans	Enterococcus	+
Unclassified	Intermediate	−
S. lactis	Lactic	−
S. cremoris	Lactic	−
S. bovis	Viridans	±
S. equinus	Viridans	−
S. salivarius	Viridans	−
S. thermophilus	Viridans	−

tell us that two bacteria are not the same. Of these five differences, however, only one is revealed by a test now commonly used in the study of streptococci. I mentioned earlier that the identity of *Streptococcus zymogenes* was not recognized over a period of time when it had attracted the attention of a number of workers on different problems. It is amusing to think that this organism would probably have been connected, in the first place, with what was already known, had some one had the wit to apply enough of the simple art of studying bacteria to determine that many of the cultures liquefied gelatin.

This picture of one of the needs of bacteriology is not attractive; it means much tedious and uninspiring labor; it is, nevertheless, the path which must be followed if order is to issue from our efforts to classify bacteria.

REFERENCES

AYERS, S. H., AND MUDGE, C. S. 1923 Streptococci of feces and mouth of cows. V. Studies of the streptococci. Jour. Inf. Dis., 33, 155–160.

HITCHCOCK, CHARLES H. 1924 Precipitation and complement fixation reactions with residue antigens in the non-hemolytic streptococcus group. Jour. Exp. Med., 40, 575–581.

LANCEFIELD, REBECCA C. 1925a The immunological relationships of *Streptococcus viridans* and certain of its chemical fractions. I. Serological reactions obtained with antibacteria sera. Jour. Exp. Med., 42, 377–395.

LANCEFIELD, REBECCA C. 1925b The immunological relationships of *Streptococcus viridans* and certain of its chemical fractions. II. Serological reactions obtained with antinucleoprotein sera. Jour. Exp. Med., 42, 397–412.

LANCEFIELD, REBECCA C. 1933 A serological differentiation of human and other groups of hemolytic streptococci. Jour. Exp. Med., 57, 571–595.

LANCEFIELD, REBECCA C. 1937 Unpublished data.

LANCEFIELD, REBECCA C., AND HARE, RONALD 1935 The serological differentiation of pathogenic and non-pathogenic strains of hemolytic streptococci from parturient women. Jour. Exp. Med., 61, 335–349.

MACCALLUM, W. G., AND HASTINGS, T. W. 1899 A case of acute endocarditis caused by *Micrococcus zymogenes* (Nov. Spec.), with a description of the microörganism. Jour. Exp. Med., 4, 521–534.

ORLA-JENSEN, S. 1919 The lactic acid bacteria. Copenhagen.

SHERMAN, J. M. 1937 The streptococci. Bact. Reviews, 1, 1–97.

SHERMAN, J. M., AND ALBUS, W. R. 1918 Some characters which differentiate the lactic-acid streptococcus from streptococci of the pyogenes type occurring in milk. Jour. Bact., 3, 153–174.

SHERMAN, J. M., AND STARK, PAULINE 1931 Streptococci which grow at high temperatures. Jour. Bact., 22, 275–285.

SHERMAN, J. M., AND STARK, PAULINE 1934 The differentiation of *Streptococcus lactis* from *Streptococcus fecalis*. Jour. Dairy Sci., 17, 525–526.

SHERMAN, J. M., AND WING, HELEN U. 1937 *Streptococcus durans* N. Sp. Jour. Dairy Sci., 20, 165–167.

SHERMAN, J. M. MAUER, J. C., AND STARK, PAULINE 1937 *Streptococcus fecalis*. Jour. Bact., 33, 275–282.

SHERMAN, J. M., STARK, PAULINE, AND MAUER, J. C. 1937 *Streptococcus zymogenes*. Jour. Bact., 33, 483–494.

SMITH, F. R., AND SHERMAN, J. M. 1938 The hemolytic streptococci of human feces. Jour. Inf. Dis., (In press).

SMITH, F. R., NIVEN, C. F., AND SHERMAN, J. M. 1938 The serological identification of *Streptococcus zymogenes* with the Lancefield group D. Jour. Bact., (In press).

THE GAS-PRODUCING SPECIES OF THE GENUS LACTOBACILLUS[1]

CARL S. PEDERSON[2]

New York State Agricultural Experiment Station, Geneva, New York

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In a previous study by the author (1936) of over four hundred strains of non-gas-producing lactic acid rods isolated from fermenting substances, the differences in characters observed appeared to be more varietal than specific in nature. It was concluded that twelve previously described and named species were identical in whole or in part with *Lactobacillus plantarum* (Orla-Jensen) Bergey *et al.*

This work has now been supplemented by a cultural study of several more or less authentic strains of previously described gas-producing species of lactobacilli and approximately 300 additional strains freshly isolated from a variety of sources. The study has included a determination of morphology, growth in various media, action on sugars, by-products of fermentation, inhibiting effects of various substances such as salt and acid, temperature limits of growth, and thermal death temperatures.

PRESENT STATUS OF THE GENUS LACTOBACILLUS

In 1919 Orla-Jensen subdivided the lactobacilli into three genera, *Thermobacterium*, *Streptobacterium* and *Betabacterium*. The first two of these groups include the non-gas-producing species which produce only traces of by-products other than lactic acid. The gas-producing species were included by Orla-

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Jensen in his genus *Betabacterium.* These produce considerable amounts of by-products other than lactic acid, such as carbon dioxide, alcohol and acetic acid. Three species are recognized by Orla-Jensen as belonging to this genus.

The fourth edition of Bergey's Manual of Determinative Bacteriology (1934) lists a total of 39 species as belonging to the genus *Lactobacillus* Beijerinck, 1901. In the key, this genus is recognized as containing three types of species which correspond in a general way with the three genera designated by Orla-Jensen. The Manual lists 19 species of gas-producing types as having been described by various investigators. It is evident, on casual examination, that some of these either are so incompletely described as to be unrecognizable, or are duplicates of previously described species. The present study was undertaken to determine the relationships involved.

PRESENT STUDIES

All of the gas-producing lactic rods vary in their morphology, with a decided tendency toward the formation of rods of short to medium length, and with rounded ends. Weakened strains or strains grown under unfavorable growth conditions, as in acid or high-alcohol-content media, tend to grow out into long filaments which often show granules when stained with methylene blue. This is true of strains isolated from fermented plant products containing acid such as sauerkraut and tomato juice, and from milk products such as kefir and the like, or alcoholic products such as beer and wine. As a result, strains have been described as distinct species on the basis of morphological differences alone. Colonies in deep agar tend to be small, compact, and round but are apt to be lenticular near the surface, or larger and spreading on the surface. On acidified or semi-solid agar, colonies are still larger and more diffuse.

When the gas-producing lactobacilli are grown under identical conditions, they produce similar end products from the fermentation of sugars. All yield approximately 45 per cent of inactive lactic acid, with possibly a slight excess of laevo or dextro lactic acid from glucose. Practically all of the remainder of the sugar

is transformed into acetic acid, alcohol and carbon dioxide. Weakened strains or strains grown under adverse circumstances such as aerobic conditions, produce less lactic acid and more acetic acid. Mannitol is a major end product from levulose, the percentage amount varying with the ability of the particular strain to utilize this carbon compound. When the pentose sugars are fermented, acetic and lactic acid are the major end products.

Characters, such as the inhibiting effect of salt and acid, utilization of protein, growth in milk, etc., are important, but since they are dependent upon the immediate past environment, they do not provide a significant basis for type differentiation.

All strains were found to ferment carbon compounds fairly consistently but the amount of fermentation naturally varied with conditions of growth. In making determinations, conditions were standardized as far as possible. Five or ten cubic centimeter amounts of a yeast-extract, Tryptone (hydrolyzed casein) broth, pH 6.8 to 7.0, containing the test compound were inoculated with one drop of a two day growth in a glucose broth. Xylose, arabinose, sucrose, and lactose solutions were usually sterilized by filtration and added to the sterilized broth. Cultures were incubated 10 days at 30 to 32°C. and then titrated with N/10 sodium hydroxide using brom-thymol-blue as an indicator.

The fermentation of arabinose was particularly interesting since a large group of cultures produced much acid while a small group failed almost entirely to utilize this sugar. The pentose, xylose, gave more variable results than any of the sugars utilized. Considerable strain differences were noted, with a variation from those that fermented relatively few to those which attacked a long series of carbon compounds. Although such fermentations have been used in the past for species differentiation in this group, it is believed that they should not be used alone for the specific designations but rather for secondary and confirming characterizations.

The non-gas-producing lactobacilli may be separated into a group that grows at low temperatures and one that grows at high temperatures. Differences in temperature range for growth

are also noted among the gas-producers and should be considered. Optimum and limiting temperatures for growth are dependent not only upon the species but upon rate of growth, completeness of fermentation, environment, and the condition of the strain. Weakened strains, for example, show narrower temperature growth limitations. The same is true in regard to thermal death temperatures. Weakened strains are killed most readily, but types which have a higher maximum growth temperature tend to be more resistant.

The optimum temperatures for growth of the various strains of gas-producing lactobacilli studied are much alike if considered from the standpoint of natural deviation from the normal. The more common strains have an optimum range from about 30° to 35°C., grow well from 18° to 40°C., show some growth at 10° and occasionally at 45°C. A second group has a slightly higher optimum range, that is, about 35° to 45°C., grows at 47° to 50°C. but grows poorly if at all, below 18°C. Intermediate between these strains are those which may grow at the higher as well as the lower temperature. A fourth but lesser known group supposedly has a lower range, usually failing to grow above 40°C. and having an optimum from about 27° to 33°C. A fifth group also has a low temperature range of growth.

Such a grouping would be of little value unless there is a correlation with other characters. This is to be found, in part, in sugar fermentations. If the sugars which show the most striking fermentation differences, i.e., arabinose, lactose, sucrose and raffinose, are selected arbitrarily as key indicators, three fermentation groups may be distinguished which are correlated with groupings based on temperature relationships. Two other groups are included in the discussion.

(1) Strains which ferment arabinose are most common. Under the conditions used in this work, these strains actively ferment sugars such as glucose, levulose and galactose but usually show little or no fermentation of raffinose, mannose, sucrose and lactose. With few exceptions they belong to the first of the temperature groups just described.

(2) In contrast to the first group, the second or higher optimum

temperature group fails to ferment or only slightly ferments arabinose, but usually ferments raffinose, mannose, sucrose, and lactose.

(3) The third, an intermediate but more uncommon group, ferments all of these sugars and its members grow over a wider range of temperatures.

(4) A fourth group will be described as the *Lactobacillus pastorianus* group. But little is known of it and cultures are not available. The strains described vary in their fermentation characters.

(5) A fifth group is similar in many respects to the first group in that it has a lower optimum temperature range and ferments arabinose; but in contrast it is very inactive toward other sugars.

There is therefore a correlation between fermentation of arabinose, lactose, sucrose and raffinose and temperature requirements for growth and three species may be recognized on the basis of these characteristics. Two additional groups need further study.

Group 1. The arabinose fermenting active group

Two hundred and forty strains of the group which ferments arabinose and generally does not ferment sucrose, lactose and raffinose, have been studied including authentic strains of *Lactobacillus brevis* (Orla-Jensen) Bergey *et al.*, *Lactobacillus pentoaceticus* Fred, Peterson, and Davenport, *Lactobacillus fermentatus* Bergey *et al.*, and *Lactobacillus lycopersici* Mickle. The 240 strains of this group were classified into three sub-groups; i.e., (*a*) sucrose negative and lactose positive, (*b*) sucrose and often lactose positive, and (*c*) sucrose and lactose negative. If this group is divided into more than one species, these sub-groups indicate the most logical method of constituting such species. The curves for frequency of acid production of these three sub-groups (fig. 1, Rows 3, 4 and 5) showed no correlation with fermentation of other sugars nor did they show any relationship to previous descriptions of species included in the study. Furthermore, the members of the three sub-groups were isolated from similar sources and showed no cultural differences. It was therefore felt that these three groups should be considered as one species. When so grouped, the frequency of acid production

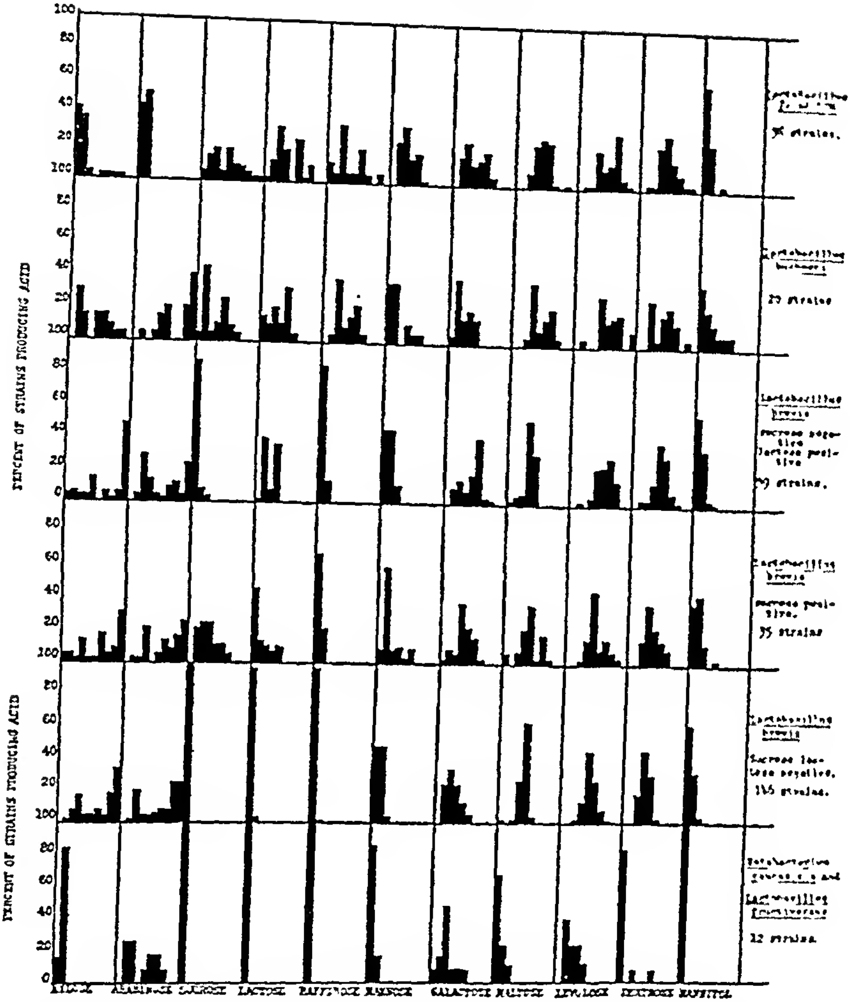

FIG. 1. FREQUENCY OF ACID PRODUCTION FROM VARIOUS CARBON COMPOUNDS BY GROUPS OF GAS-PRODUCING STRAINS OF THE GENUS LACTOBACILLUS EXPRESSED AS PER CENT OF STRAINS PRODUCING FROM 0 TO 9 CC. OF N/10 ACID IN 10 CC. OF MEDIUM

Ten spaces used for each sugar signify in the first block per cent of strains producing no acid, the second, per cent of strains producing 0.1 to 1 cc. of N/10 acid, the third space 1.1 to 2.0 cc., etc. The tenth space is used for percentage of strains producing 8.1 to 12.5 cc.

from carbon compounds shows a more or less normal series of curves (figs. 2A and 2B). For this reason, it is felt that the group of arabinose-fermenting, gas-producing lactobacilli which

generally do not ferment raffinose, mannose, sucrose, and lactose should be considered as one species.

From a study of the literature it appears that the description of the species *Betabacterium breve* Orla-Jensen, 1919, syn. *Lactobacillus brevis* Bergey *et al.*, is the first one given for a recognizable member of this group. Authentic strains of *Bacillus brassicae fermentatae* Henneberg, syn. *Lactobacillus fermentatus* Bergey *et al.*, *Lactobacillus pentoaceticus* Fred, Peterson and Davenport,

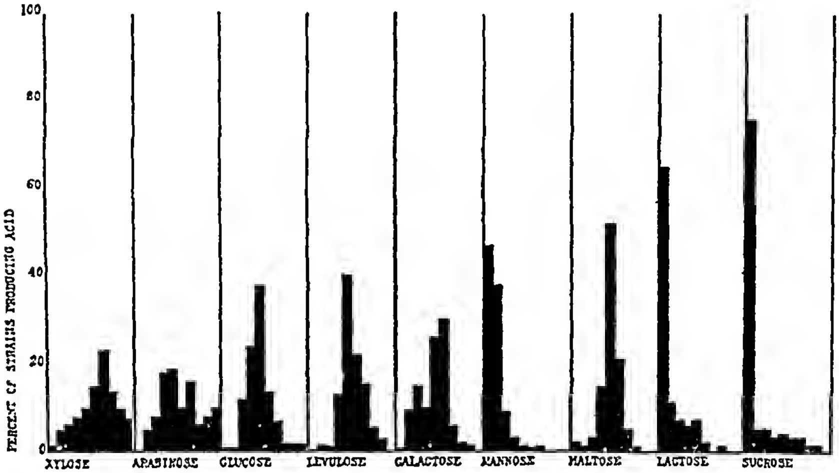

FIG. 2A. FREQUENCY OF ACID PRODUCTION FROM VARIOUS CARBON COMPOUNDS BY CERTAIN GAS PRODUCING STRAINS OF THE GENUS LACTOBACILLUS EXPRESSED AS PER CENT OF STRAINS PRODUCING FROM 0 TO 9 CC. OF N/10 ACID IN 10 CC. OF MEDIUM

Ten spaces used for each sugar signify in the first block per cent of strains producing no acid, the second, per cent of strains producing 0.1 to 1 cc. of N/10 acid, the third space 1.1 cc. to 2.0, etc. The tenth space is used for percentage of strains producing 8.1 to 12.5 cc.

and *Lactobacillus lycopersici* Mickle, have been studied in comparison with *Lactobacillus brevis* and are considered identical. The first name is a trinomial and thus cannot be considered from the standpoint of rules of nomenclature. The other two species names were proposed subsequent to the work of Orla-Jensen.

Certain non-authentic strains of *Bacillus acidophil-aerogenes* Torrey and Rahe syn. *Lactobacillus acidophil-aerogenes* Bergey *et al.* were studied and considered identical; but since the char-

acters of these strains did not correspond with those given in the original published description of Torrey and Rahe, it is difficult to place this species. As characterization is incomplete, the name given by Torrey and Rahe is perhaps best considered as a possible or probable synonym of *Betabacterium breve*.

Published descriptions of *Bacillus panis fermentati* Henneberg, syn. *Lactobacillus panis* Bergey *et al.* indicate that this organism

FIG. 2B. FREQUENCY OF ACID PRODUCTION FROM VARIOUS CARBON COMPOUNDS BY CERTAIN GAS PRODUCING STRAINS OF THE GENUS LACTOBACILLUS EXPRESSED AS PER CENT OF STRAINS PRODUCING FROM 0 TO 9 CC. OF N/10 ACID IN 10 CC. OF MEDIUM

Ten spaces used for each sugar signify in the first block per cent of strains producing no acid, the second, per cent of strains producing 0.1 to 1 cc. of N/10 acid, the third space 1.1 to 2.0 cc., etc. The tenth space is used for percentage of strains producing 8.1 to 12.5 cc.

is identical with the cultures included in this group and therefore these names are also regarded as synonyms of *Betabacterium breve*.

The same condition may hold true for *Bacterium soya* Saito syn. *Lactobacillus soya* Bergey *et al.* The description given for this species is too incomplete to permit identification.

A brownish red pigmented variety of this arabinose-fermenting group has recently been isolated by Davis and Mattick and received under a name which indicates that they regard it as belonging to this group. This conclusion has been confirmed by our own studies.

Group 2. The arabinose non-fermenting group

For the second group of organisms, which ordinarily grow at a higher temperature range, the name *Lactobacillus fermentum* Beijerinck is suggested. Thirty-six strains have been included in this group and as noted (fig. 1, Row 1), they usually do not ferment arabinose nor xylose but generally ferment sucrose, lactose and raffinose. These types have been well described by Beijerinck (1901) and later by Smit (1916) as *Lactobacillus fermentum*. Still later, Orla-Jensen named similar organisms *Betabacterium longum* syn. *Lactobacillus longus* Bergey *et al.*

Two other described species are regarded as identical with this species. These are *Bacterium gayonii* Müller-Thurgau and Osterwalder syn. *Lactobacillus gayonii* Pederson, which is believed to be similar to Gayon and Dubourg's mannitol forming rod from wine; and *Bacterium intermedium* Müller-Thurgau and Osterwalder, syn. *Lactobacillus intermedium* Bergey *et al.*

Group 3. The intermediate group

A third intermediate group of arabinose-fermenting, gas-producing lactobacilli includes the strains which are more active toward other sugars (fig. 1, Row 2) than is the *Lactobacillus brevis* group. They are more heat resistant and show a wider temperature growth range. This group includes three described species but authentic strains are not available for all of these species. It is therefore difficult to determine the correct name or names for the described species of this group. The description of the species *Bacillus buchneri* Henneberg (1903) syn. *Lactobacillus buchneri* Bergey *et al.* although fairly complete is not sufficiently inclusive. This description is therefore emended and the name suggested as the proper designation for the entire intermediate group.

The remaining species are *Bacillus wehmeri* Henneberg, syn. *Lactobacillus wehmeri* Bergey *et al.*; and *Bacillus hayduckii* Henneberg, syn. *Lactobacillus hayduckii* Bergey *et al.* which were described in the same paper. They are very closely related types and may be considered by some to be identical with *Lactobacillus buchneri*. *Bacterium mannitopoeum* Müller-Thurgau syn. *Lactobacillus mannitopoeus* Pederson from published descriptions also

appears to belong to this group. However, a freshly isolated strain from Osterwalder (1928) does not have the characters given in the original description.

Group 4. The Lactobacillus pastorianus group

A number of species described as long rods with a lower temperature growth range than other types of lactobacilli are described in the literature but few authentic strains are available at present. *Saccharobacillus pastorianus* Van Laer, syn. *Lactobacillus pastorianus* Bergey *et al.* is quite completely described. Closely related to this organism but less active in the fermentation of sugars are *Saccharobacillus pastorianus* var. *berolinensis* Henneberg, *Bacillus fusciformis* Schönfeld and Rommel and *Bacillus lindneri* Henneberg, syn. *Lactobacillus lindneri* Bergey *et al.* Because of the meagre information now available it is difficult definitely to establish the relationships of these types.

Group 5. The inactive group

A less active type of gas-producing lactobacilli to which Orla-Jensen (1919) applied the name *Betabacterium caucasicum* was isolated by him from kefir grains. Orla-Jensen believed this species to be the organism to which Kern (1882) referred in his original description of *Dispora caucasicum*. Several cultures similar to those described by Orla-Jensen have been isolated from kefir grains received from Dr. L. A. Rogers of Washington. These ferment arabinose, galactose, maltose and levulose and sometimes glucose but do not attack other sugars (fig. 1, Row 6).

A culture labeled *Bacterium mannitopoeum* Müller-Thurgau was received from Osterwalder. This culture has much in common with the inactive group in that it is quite inactive toward sugars; but its characteristics do not agree with the previously published description of the species described by Müller-Thurgau. These descriptions indicate that the species is related to *Lactobacillus buchneri* while this new culture is similar to *Lactobacillus fructivorans* Charlton, Nelson and Werkman. On the other hand, the culture sent by Osterwalder as well as authentic strains of the latter organism are similar to strains of *Betabacterium*

caucasicum Orla-Jensen in that they ferment sugars poorly, attack arabinose, galactose, maltose, and levulose only (fig. 1, Row 6), and are furthermore, difficult to isolate and to carry in pure culture. It is doubtful as yet whether the group should be considered as a single species.

CONCLUSION

Since the strains of gas-producing lactobacilli are all more or less related to each other, and strains intermediate between the various groups, can be obtained it appears that the most usable key to species is one based on a combination of characters. It appears that growth temperatures and action on arabinose followed by action on lactose, sucrose, and raffinose, are the most important differential characters for organisms in this group.

Upon the basis of these characters, four species may be described as follows:

Lactobacillus brevis (Orla-Jensen) Bergey *et al.*

Synonyms: *Bacillus brassicae fermentatae* Henneberg, *Bacillus panis fermentati* Henneberg, *Lactobacillus pentoaceticus* Fred, Peterson and Davenport, *Lactobacillus lycopersici* Mickle, and possibly *Bacterium soya* Saito, and *Bacillus acidophil-aerogenes* Torrey and Rahe.

Morphology: Non-motile, gram-positive rods, ordinarily 0.6 to 0.8 by 1.0 to 4.0 microns, with rounded ends occurring singly or in short chains and occasionally in long filaments which may show granulation.

Gelatin colonies: No growth.

Yeast-extract-glucose stab gelatin: Filiform growth. No liquefaction.

Litmus milk: Usually acid but seldom curdled or reduced.

Agar slant: Growth, if any, faint.

Broth: Turbid, clearing after a few days.

Potato: Growth, if any, scanty.

Does not attack casein as a rule.

Acid formed in arabinose, xylose, levulose, glucose, galactose, and maltose. Strains vary in fermentation of lactose, sucrose, mannose, raffinose and mannitol. Salicin, sorbitol, glycerol, rhamnose, dextrin, inulin and starch seldom fermented. They usually show a particularly vigorous fermentation of arabinose.

Lactic acid, usually inactive; acetic acid, ethyl alcohol and carbon

dioxide formed in fermentation of aldohexoses. Mannitol produced from levulose. Acetic and lactic acid produced from pentoses.

Microaerophilic.

Optimum temperature 30° to 35° C., maximum 40° to 45°C., minimum 10° to 15°C.

Sources from which isolated: Cheese, milk, feces, fermenting potatoes, sauerkraut, ensilage, manure, soil, sour dough, and spoiled tomato products.

Habitat: Widely distributed, particularly in fermenting plant and animal products.

Lactobacillus fermentum Beijerinck.

Synonyms: *Betabacterium longum* Orla-Jensen, *Bacterium gayoni* Müller-Thurgau and Osterwalder, *Bacterium intermedium* Müller-Thurgau and Osterwalder.

Morphology: Non-motile, gram-positive rods ordinarily 0.5 to 1.0 by 1.0 to 15.0 microns sometimes in pairs or short chains or filaments.

Yeast-extract-glucose gelatin: Filiform growth. No liquefaction.

Agar colonies: Flat, circular, small.

Agar slant: Growth, if any, very scant.

Broth: Turbid, clearing after several days.

Litmus milk: Unchanged or slightly acid but seldom curdled or reduced.

Potato: No growth.

Acid usually formed in glucose, levulose, mannose, galactose, sucrose, maltose, and raffinose. Some strains ferment xylose. Usually does not ferment arabinose, rhamnose, sorbitol, mannitol, inulin, dextrin, starch and salicin.

Lactic acid, usually inactive; acetic acid, ethyl alcohol, and carbon dioxide are formed in the fermentation of aldohexoses. Mannitol is formed in the fermentation of levulose. Acetic and lactic acids are produced from pentoses if they are fermented.

Microaerophilic.

Optimum temperature 35° to 40°C. or higher, maximum 48° to 50°C., minimum 15° to 18°C.

Sources from which isolated: Yeast, milk products, fermenting dough, potatoes, or vegetables, spoiled tomato products and wine.

Habitat: Widely distributed in nature particularly in fermenting plant or animal products.

Lactobacillus buchneri (Henneberg) Bergey *et al. emend.* Pederson

Synonyms: *Bacillus wehmeri* Henneberg, *Bacillus hayduckii* Henneberg and *Bacterium mannitopocum* Müller-Thurgau.

Morphology: Non-motile, gram-positive rods, ordinarily 0.5 to 0.7 by 1.0 to 4.0 microns, occurring singly or in chains or long filaments. Granulation has not been demonstrated.

Yeast-extract-glucose stab gelatin: Filiform growth. No liquefaction.

Litmus milk: Usually unchanged but may be slightly acid but with no reduction.

Agar slant: Growth, if any, faint.

Broth: Turbid, clearing after several days.

Potato: Growth, if any, scanty.

Acid usually formed in arabinose, xylose, glucose, levulose, galactose, mannose, sucrose, lactose, maltose, and raffinose. Mannitol, sorbitol, glycerol, rhamnose, salicin, inulin, dextrin, or starch fermented by a few strains. (A restatement of the original description.)

Lactic acid, usually inactive; acetic acid, ethyl alcohol and carbon dioxide formed in fermentation of aldohexoses. Mannitol produced from levulose, acetic and lactic acid produced from pentoses. (A more complete statement than the original description.)

Microaerophilic.

Optimum temperature 32° to 37°C., maximum 44° to 48°C., minimum 10° to 15°C.

Sources from which isolated: Sour mash, molasses, wine, tomato catsup and sauerkraut.

Habitat: Widely distributed in fermenting substances.

Lactobacillus pastorianus (Van Laer) Bergey *et al.*

Synonyms: *Saccharobacillus pastorianus* var. *berolinensis* Henneberg, *Lactobacillus berolinensis* Bergey et al., *Bacillus fusciformis* Schönfeld and Rommel and *Bacillus lindneri* Henneberg.

Morphology: Non-motile, gram-positive rods, ordinarily 0.5 to 1.6 by 2.0 to 20.0 microns, occurring singly or in chains.

Agar colonies: Small gray.

Agar slant: Slow growth.

Broth: Turbid.

Litmus milk: Usually acid.

Acid usually formed in arabinose, glucose, levulose, galactose, mal-

tose, sucrose, raffinose, and mannitol. Weaker strains may not ferment all these carbon compounds. Forms carbon dioxide, alcohol, and acetic and lactic acid, from sugar.

Microaerophilic.

Optimum temperature 27° to 32°C.

Sources from which isolated: Sour beer and distillery yeast

Habitat: Probably more widely distributed than is indicated by the isolations made.

The relationships of the species *Lactobacillus fructivorans* Charlton, Nelson and Werkman and *Betabacterium caucasicum* Orla-Jensen are still uncertain. More complete information is needed in regard to their relationships to the forementioned species and more definite knowledge should be obtained with respect to their gas-producing properties. The characteristics of organisms found in kefir grains should also be determined more completely.

REFERENCES

Beijerinck, W. 1901 Sur les ferments lactiques de l'industrie. Arch. Néerland de Sciences Exactes et Naturelles, Sér. 11, 7, 212-243.

Bergey, D. H. 1934 Manual of Determinative Bacteriology, 4th Edition, 664 pp. Baltimore.

Henneberg, W. 1903 Zur Kenntnis der Milchsäurebakterien der Brennereimaische, der Milch, des Bieres, der Presshefe, der Melasse, des Sauerkohls, der sauren Gurken und des Sauerteigs, sowie einige Bemerkungen über die Milchsäurebakterien des menschlichen Magens. Cent. f. Bakt., II Abt., 11, 154-170.

Kern, E. 1882 Ueber ein neues Milchferment aus dem Kaukasus. Bull. de la Soc. Imp. des Naturalistes de Moscow, 56, 141-173.

Orla-Jensen, S. 1919 The lactic acid bacteria. Mém. de l'Acad. Roy. d. Sci. et d. Lettres de Danemark, Copenhague. Sect. d. Sci., 8 Sér., 5, No. 2.

Pederson, C. S. 1936 A study of the species *Lactobacillus plantarum* (Orla-Jensen) Bergey et al. Jour. Bact., 31, 217-224.

Smit, J. 1916 Studien über *Lactobacillus fermentum* (Beijerinck). Zeitschr. f. Garungsphysiol., 5, 273-293.

Van Laer 1892 Contributions a l'Histoire des Ferments des Hydrates de Carbone. Acad. Roy. de Belge, Memoires Couronnés, 47.

THE GROWTH OF YEAST IN A MAGNETIC FIELD

GRACE C. KIMBALL

Laboratory of Bacteriology, College of Agriculture, Cornell University, Ithaca, New York

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In regard to magnetism, all substances may be divided into three classes, namely, ferro-, para-, and dia-magnetic. Ferro- and para- magnetic substances offer less resistance (reluctance) to the lines of force of the magnetic field than the air, therefore the lines of force tend to be drawn into the substance. A sliver of such material tends to turn so that its long axis is in the direction of the field. In a heterogeneous field the material will be moved toward the stronger part of the field. These characteristics are easily observed in iron, but the motion of paramagnetic substances is so slight that it can be detected only by microscopic methods. Diamagnetic substances, contrary to the first two classes, offer more reluctance to the magnetic field than does the air. A sliver of such material tends to turn across the lines of force, and to be forced out of a heterogeneous field.

Of the few papers proving an influence of a magnetic field upon living organisms, the most extensive refer to protoplasmic streaming. If this is an electrical phenomenon, it should be affected by a magnet. After several investigators had failed to find an effect, Ewart (1903) by using a stop-watch and an ocular micrometer, finally detected a retardation of the rate of streaming. He also found that certain elongated plant cells were paramagnetic; they swung in the direction of the applied magnetic field.

Ssawostin (1930a) confirmed Ewart's findings, using algae. He could accelerate or retard the rate of streaming, depending upon the direction of the applied field. He attributed retardation to friction between the protoplasm and the cell walls,

produced as the cellular electrical current attempted to turn toward a stable position in regard to the applied magnetic field and carried the protoplasm with it. Acceleration, he attributed to an increased rate of chemical reaction.

Ssawostin (1930b) also found that he could stimulate the growth of the first leaf of a wheat seedling by applying a magnetic field to its base. This is the only paper found reporting an effect on the growth of plants.

Chévenau and Bohn (1903) reported that the growth of Protozoa was inhibited by a magnetic field. They used 5000 or 8000 gauss for 3 to 5 days, and got quite pronounced effects.

Bacteria and yeast have been used unsuccessfully as test organisms. Leusden (1929) found that the same number of colonies developed on exposed plates as on controls. Jennison (1937) reported that a homogeneous field of 3000 gauss did not affect the "size of colony, size and shape of individual cells, reaction to gram stain, pigment and spore production." In view of the work on protoplasmic streaming it would seem that these writers were looking for too large and obvious an effect. The work to be described also indicates that the use of a homogeneous field might be responsible for negative results in some cases.

METHOD

A Burgundy wine yeast was used. The technique was that of Tuthill and Rahn (1933). An agar plate was flooded with a suspension of 24-hour-old yeast cells, free from buds, and the liquid was poured off after 1 minute. There should be about 50 yeast cells per microscopic field (400× magnification) left on the surface. Malt extract agar, and 30°C., were used.

The standard method of exposure was the following: When the culture was 1.5 hours old, the Petri dish was inverted, resting on a cardboard frame. The horseshoe magnet was clamped below it, the poles pointing upward, and protruding through a slit in the cardboard. This opening was opposite the center of the plate. The poles were 8 mm. from the agar surface. The exposure lasted for 30 minutes. After incubation for another half hour, the yeast, now 2.5 hours old, and showing usually between 10 and 20 per cent buds, was etherized to stop growth.

Unless otherwise specified, the exposures were made in this manner.

Figure 1 shows how the counting was done. The shaded areas indicate the parts of the agar directly above the poles of the magnet. The strip of agar in line with these shaded areas was removed to a microscope slide. Counts were made on the agar at each of the vertical dotted lines. Successive fields along

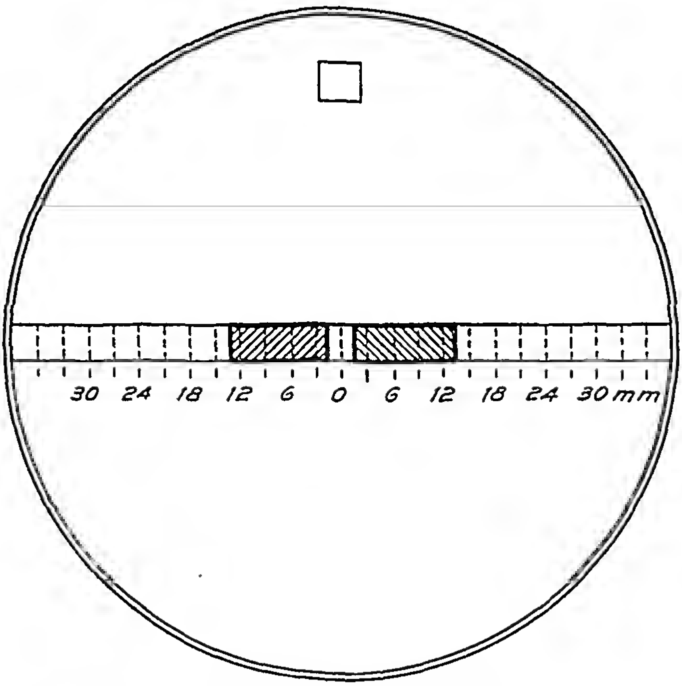

Fig. 1. Petri Dish Showing the Position of the Magnetic Poles, and the Section of Agar Used for Counting the Yeast Buds at Intervals of 3 mm.

each dotted line were examined until the number of buds on 500 cells had been determined.

EXPERIMENTAL RESULTS

Typical results were obtained with a small horseshoe magnet, which exerted a force of 4 gauss[1] at a point 1 cm. distant from

[1] Measurement of field strength through the courtesy of Dr. Tomboulian of the Physics Department of Cornell University.

the ends of the poles in the midline of the magnet. The poles together measured 25 mm. from end to end. They were 4 mm. wide, and were separated at the end by 3 mm. The magnet was 100 mm. long at the midline. The shape of the magnet is important since it alters the shape of the field. Each magnet must be tested to find the distance from the yeast that is most effective. The magnet used gave good results at 8 and 3 mm. from the cells. Table 1 shows the data obtained when the magnet was 8 mm. distant (Series I), and 3 mm. distant (Series II).

TABLE 1

Each number represents the number of buds on 500 yeast cells, under the poles of magnet A 4, and outside: Each count is 3 mm. distant from its neighbors.

	OUTSIDE THE POLES					UNDER THE POLES									OUTSIDE THE POLES								
	Series I: Distance from magnet 8 mm.																						
1				43	43	39	35	26	28	15	25	28	30	31	40	50	57						
2						94	95	113	79	69	55	52	63	62	80	104	108	106	100				
3	85	67	65	61	68	58	44	57	53	60	61	49	70	79	72	74	75	78	80				
4	88	70	69	58	66	49	54	56	65	49	37	53	52	59	62	60	61	67	70	64	89		
5			60	64	57	54	44	45	41	37	43	62	56	49	64	72	68	74	63	70	77		
6		52	54	45	49	40	36	26	26	38	32	31	27	43	42	56	48	52	53	49	44	56	50
7						61	—	—	53	52	55	44	65	51	50	55	67	61	61				
8	82	77	66	79	71	66	69	84	57	50	58	81	67	76	69	62	72	76	77	91	97	98	91
	Series II: Distance from magnet 3 mm.																						
1				50	38	26	29	25	36	47	30	28	33	28	36	44	47						
2				67	72	54	39	39	52	52	54	49	54	60	68	67							
3			57	61	62	66	66	36	47	—	43	41	37	50	51	57	57	55					
4		64	65	60	51	—	43	45	48	35	44	55	31	45	47	36	46	59	55	68	62		

Each number represents the number of buds on 500 yeast cells. The central part of the table shows the number of buds under the poles of the magnet. The outer numbers are those counts made outside of the poles, where the growth is normal. Each count was made 3 mm. distant from the neighboring counts. In all of the experiments the average number of buds under the poles is lower than that outside, indicating that the budding was retarded by the magnet.

This can be shown graphically by plotting the percentage of buds against the distance from the center of the magnet at which

the counts were made. These percentages have been averaged for Series I and II, and the two curves in figure 2 show the composite result of all of these experiments.

The probable error (table 2) was computed from the formula

$$\text{P.E.} = 0.6745 \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

In all cases except one, the decrease in the amount of yeast buds is more than 3 times the probable error, i.e., it is a statistically significant decrease.

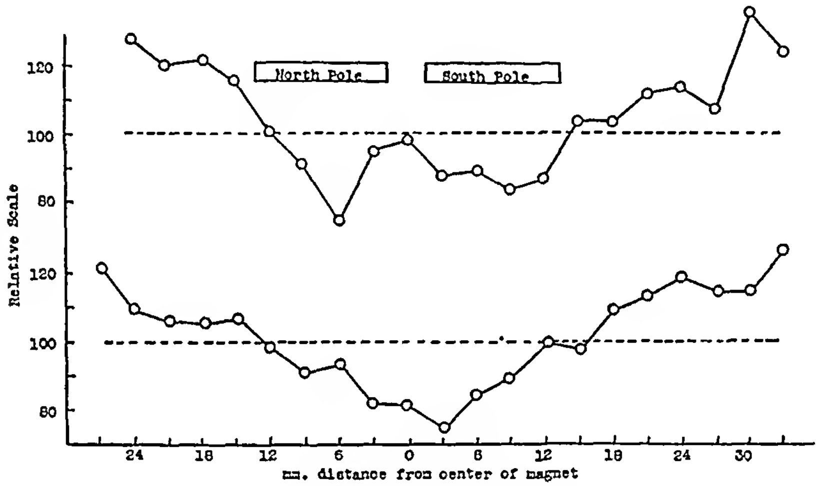

FIG. 2. AVERAGE CURVES. SERIES I AND II
Above: Average of 4 experiments, magnet 3 mm. from cells. Series II.
Below: Average of 8 experiments, magnet 8 mm. from cells. Series I.

The possibility must be investigated that this inhibition might be caused by some distant physical or chemical action of the steel itself upon the yeast (table 3).

To determine the effect of the steel without the magnetic field, the oval, non-magnetic end of the horseshoe was filed off straight, and clamped below the yeast cells in the same manner as the poles had been previously. The resultant curve showed only the normal fluctuation; the slight decrease in budding is not larger than the probable error (table 4).

When the magnetic field was cut off by a soft iron "keeper" which fitted across the poles of the magnet, the yeast was not inhibited (table 4, no. 2). Neither the "distant" action of the iron nor of the sides of the magnet influenced the rate of budding. If, however, a small iron cap was placed over each pole of the magnet (table 4, no. 5), the inhibitory effect was very evident. The magnetic field was not altered.

Two experiments in which a glass microscope slide was placed between the magnet and the yeast resulted in inhibition. This

TABLE 2

Computation of probable errors for table 1

	Mean number of buds and probable error		Difference and probable error of difference	Difference divided by probable error of difference
	Outside poles	Under poles		
Series I				
1	44.0 ±2.23	28.2 ±1.89	15.8 ±2.94	5.37
2	99.6 ±3.08	75.7 ±4.43	23.9 ±5.4	4.4
3	72.5 ±1.49	59.0 ±2.24	13.5 ±2.69	5.02
4	68.0 ±1.62	52.7 ±1.64	15.3 ±2.33	6.57
5	67.4 ±1.25	48.4 ±1.66	19.0 ±2.08	9.13
6	50.0 ±0.805	33.0 ±1.34	17.0 ±1.56	10.8
7	58.8 ±1.67	54.4 ±1.84	4.4 ±2.48	1.77
8	79.1 ±1.975	67.5 ±2.38	11.6 ±3.1	3.7
Series II				
1	43.0 ±1.6	31.8 ±1.4	11.2 ±2.14	5.2
2	68.5 ±0.69	50.3 ±1.5	18.2 ±1.65	11.03
3	56.0 ±0.95	47.0 ±2.62	9.0 ±2.8	3.2
4	55.0 ±1.83	43.0 ±1.66	12.0 ±2.48	4.84

eliminated physical effects other than magnetism, but left the magnetic field normal.

A very simple method for avoiding chemical or "distant" effects, other than magnetic, is to coat the magnet with paraffin. The poles were dipped in melted paraffin, removed, and inverted. The resulting curve showed not only the usual inhibition of budding in the center, but also a small "secondary" inhibition on either side. Then a block of paraffin was placed across the

TABLE 3

Series III. Elimination of influences other than magnetism

	METHOD OF EXPOSURE	OUTSIDE THE POLES								UNDER THE POLES									OUTSIDE THE POLES					
1	Oval end of horseshoe					45	45	50	47	43	38	50	47	54	37	40	43	39	53	46	44	51		
2	Soft iron strip connecting both poles					88	73	79	74	90	78	73	88	79	81	67	93	83	93	86	92	104		
3	Glass shield	61	83	85	90	92	83	87	79	70	73	69	76	60	61	75	61	95	75	87	81	82	91	
4	Glass shield				106	93	100	115	104	98	89	84	70	81	73	78	95	109	103	105	97	100	101	103
5	Separate galvanized iron cap on each pole				110	120	116	116	128	106	122	109	88	79	107	99	119	114	128	124	135	123	122	128
6	Galvanized iron strip, without magnet			107	76	91	114	95	92	101	115	110	109	85	106	92	83	92	107	85	80	99	112	

ends of the poles, like a keeper. The curves (fig. 3) again showed the secondary inhibitions. This verified the supposition that when the poles were dipped there was a thicker layer of paraffin

TABLE 4

Series III. Computation of probable errors for table 3

	METHOD OF EXPOSURE	MEAN NUMBER OF BUDS AND PROBABLE ERROR		DIFFERENCE AND PROBABLE ERROR OF DIFFERENCE	DIFFERENCE DIVIDED BY PROBABLE ERROR OF DIFFERENCE
		Outside poles	Under poles		
1	Oval end of horse-shoe	45.4 ±0.792	44.1 ±1.52	1.3 ±1.71	0.765
2	Soft iron strip connecting both poles	85.5 ±1.43	81.1 ±1.99	4.4 ±2.44	1.8
3	Glass shield	83.4 ±1.185	71.0 ±2.31	12.4 ±2.6	4.8
4	Glass shield	102.2 ±0.914	86.3 ±2.69	15.9 ±2.84	5.6
5	Separate galvanized iron cap on each pole	122.5 ±1.38	107.1 ±3.17	15.4 ±3.45	4.5
6	Galvanized iron strip, without magnet	94.8 ±2.21	99.2 ±2.47	−4.4 ±3.31	−1.33

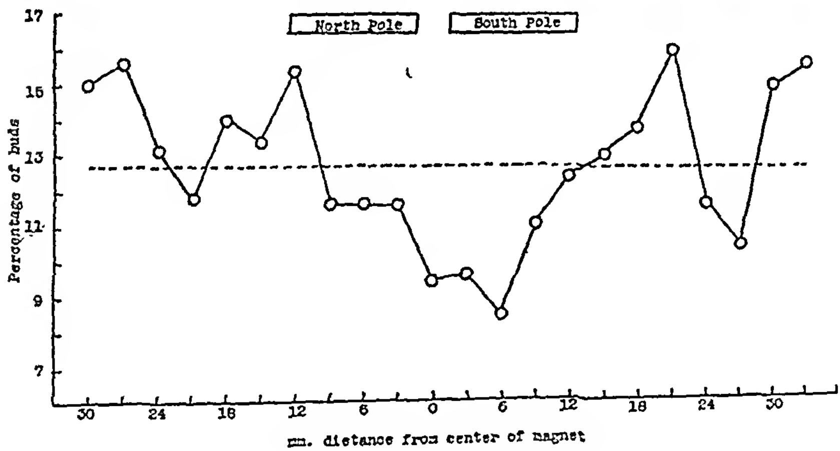

FIG. 3. RESULT OF EXPOSURE TO A MAGNET WHICH HAS A BLOCK OF PARAFFIN ACROSS ITS POLES

across the ends than on the sides. Paraffin is much more resistant to the passage of lines of force than is the air. Therefore, some of the lines of force passed out of the side of the pole (rather than out of the end) through the thinner layer of paraffin (or,

in the case of the block, through none at all) to get to the air in the shortest distance. This meant that the fields opposite the outer edges of the poles were not as strong as normally, and that outside of this there was an area of increased strength, which was responsible for the secondary inhibition. These phenomena were observed in all of 6 experiments.

The yeast so far had been exposed only when 1 to 1.5 hours of age. The other stages of development were now investigated for their sensitivity to magnetism (table 5). Exposure during the first hour was without effect. Between 1 and 2 hours of age

TABLE 5

Series V. Results of experiments planned to determine at what age the yeast are sensitive to the action of the magnetic field

	PERIOD OF EXPOSURE	MEAN NUMBER OF BUDS AND PROBABLE ERROR		DIFFERENCE AND PROBABLE ERROR OF DIFFERENCE	DIFFERENCE DIVIDED BY PROBABLE ERROR OF DIFFERENCE
		Outside poles	Under poles		
	hours				
1	0 -0.5	55.6 ±0.709	54.1 ±1.01	1.5 ±1.23	1.22
2	0.5-1	61.2 ±1.56	60.6 ±0.938	0.6 ±1.82	0.33
3	0 -1	64.1 ±1.09	60.0 ±2.1	4.1 ±2.6	1.58
4	1 -1.5	93.3 ±1.89	71.3 ±1.26	12.0 ±2.27	9.7
5	1 -1.5	77.6 ±1.34	64.5 ±1.96	13.1 ±2.30	5.5
6	1 -2	63.4 ±1.81	50.4 ±1.1	13.0 ±2.12	6.1
7	1 -2	53.2 ±0.962	47.5 ±1.69	5.7 ±1.94	2.94
8	2 -2.5	94.6 ±1.39	87.5 ±2.01	7.1 ±2.44	2.91
9	2 -2.5	83.7 ±0.701	88.0 ±0.963	4.3 ±1.19	−3.61
10	2 -2.5	63.5 ±1.24	66.0 ±1.24	2.5 ±1.75	−1.43

the yeast could be inhibited. Exposure after 2 hours produced no effect. The sensitive stage occurs during the last half of the lag phase, since the buds just start to appear at 2 hours, or soon thereafter. These ages, of course, apply only to a culture which shows between 10 and 20 per cent buds when it is 2.5 hours old.

It seemed possible that the thirty minutes exposure used in previous experiments might be longer than necessary (table 6). When exposure time was shortened to 25 and 20 minutes, there was still inhibition. Exposures of 17, 15, and 10 minutes were insufficient to affect the yeast. Peculiarly enough, an even shorter exposure, 5 minutes, was capable of inhibiting budding.

Lengthening the exposure time to 60, 120, and 150 minutes did not interfere with the inhibitory effect.

All of these experiments were made in a heterogeneous magnetic field. Through the courtesy of Dr. Gibbs, of the Physics Department, a homogeneous field of 11,000 gauss, supplied by the pole pieces of the cyclotron magnet, was used for one series of experiments. Plates 1.5 hours old were exposed to this strong field, which acted perpendicularly to the surface of the agar. A plate was removed after 5, 10, 20, 40, and 80 minutes of exposure,

TABLE 6

Series VI. Effect of varying the length of time of exposure

	LENGTH OF EXPOSURE TIME	MEAN NUMBER OF BUDS AND PROBABLE ERROR		DIFFERENCE AND PROBABLE ERROR OF DIFFERENCE	DIFFERENCE DIVIDED BY PROBABLE ERROR OF DIFFERENCE
		Outside poles	Under poles		
	min.				
1	5	78.3 ±1.54	59.0 ±1.64	19.3 ±2.24	8.6
2	5	165.0 ±3.09	127.0 ±1.97	38.0 ±3.67	10.4
3	10	52.0 ±1.0	56.2 ±1.48	−4.2 ±1.79	−2.32
4	10	72.3 ±0.401	72.8 ±1.79	−0.5 ±1.83	−0.273
5	10	44.5 ±1.62	46.0 ±0.72	1.5 ±1.77	0.85
6	15	77.0 ±1.82	74.4 ±1.75	2.6 ±2.52	1.03
7	15	84.0 ±4.93	79.5 ±3.78	4.5 ±6.2	0.73
8	17	81.0 ±1.89	92.6 ±1.38	−11.6 ±2.34	−4.96
9	17	127.0 ±2.84	126.0 ±2.45	1.0 ±3.75	0.266
10	20	78.2 ±2.02	66.8 ±1.68	11.4 ±2.63	4.3
11	25	73.3 ±0.824	50.3 ±3.35	23.0 ±3.44	6.7
12	150	78.0 ±1.12	57.0 ±1.29	21.0 ±1.71	12 3
13	150	82.2 ±1.97	64.4 ±1.65	17.8 ±2.56	6.96

and etherized when 2.5 hours old. The number of yeast buds on 500 cells was, respectively, 68, 56, 55, 63, and 81, with 63 on the control plate. Each number is the average of 4 counts. This shows very plainly that there was no inhibition in a field 3000 times as strong as that obtained with the small horseshoe magnet. The higher count on the 80-minute plate was probably individual variation. It seems unlikely that an effect would be apparent only after 80 minutes exposure and not before, and it also seems unlikely that it would be a stimulation. However, this could be proved only by a great number of experiments.

DISCUSSION

The experiments in a homogeneous field, in addition to an incomplete group of experiments with bar magnets, and with iron shields to distort the earth's field (Kimball), disclosed an important principle: The effect is not produced by a magnetic field as such, but by a change in field strength, a field gradient. This explains why many biological experiments with strong homogeneous fields failed, while the very weak heterogeneous field of a small horseshoe magnet produced statistically sound results.

Although this principle was first noticed in connection with another group of experiments, it can be seen to apply to the experiments with a horseshoe magnet equally as well. In these experiments the field is strongest in the center of the plate, and becomes continuously weaker toward the sides. The percentage of buds rises as the field strength decreases. With paraffin across the poles there is a break in the field strength, and a corresponding break in the curve. The field along the axis line of a bar magnet drops rapidly, and there is a difference in the percentage of yeast buds. The negative results obtained with the homogeneous field of the cyclotron magnet are particularly significant in regard to this principle.

The fact that a heterogeneous field is necessary to inhibit budding suggests a possible mechanism by which this inhibition might be produced. Homogeneous and heterogeneous fields differ in their action on paramagnetic substances. A homogeneous field orients a sliver of such material, while a heterogeneous field not only orients, but also tends to force the material into the stronger part of the field. Since only a heterogeneous field produced biological effects, it would seem possible that the effect depended upon a translocation of some of the cellular elements. This might be imagined in the following way: The new protoplasm for the bud is built up step by step, probably by a series of catalysts in some orderly fashion (Rahn). Certain free radicals, such as $(C_6H_5)_3C$—, OH, and O, are known to be much more strongly paramagnetic than most substances, and some cell catalysts contain iron. It may be that some such substance, quite strongly magnetic, is pulled out of line toward

the stronger part of the field, thus interrupting the order, and thereby the metabolic process, and thus retarding budding. There would be retardation only in those cells lying in such a position that the strongest part of the field was perpendicular to the path of metabolism. Since the cells were distributed at random, this agrees with the fact that only 20 to 30 per cent of the cells were affected. A further check on this hypothesis would be afforded by experiments in a liquid culture, in which the cells are free to move. A few experiments have been made, but the variation between different suspensions and the error of sampling make the results of little value until a great many experiments have been made.

Although a stronger field than that used is necessary to move paramagnetic substances enough so that the motion can be seen, *it must be realized that a movement over the distance of 10 Å.* = 0.000,000,1 cm., would be sufficient to move a molecule out of the sphere of neighboring molecules, and thus produce the effect in the cell.

Another less probable explanation depends upon the findings of Pissarschewsky, and Pissarschewsky and Rosenberg, that ferro- and para-magnetic metals dissolve more slowly in acids in a magnetic field of 2500 gauss. Diamagnetic metals dissolve more rapidly. Bhatnagur, Nathur, and Kapur also found that the rate of oxidation-reduction reactions, as well as the solution of metals, could be influenced by a magnetic field. It is not likely that this fact has much bearing on the inhibition of budding, since these chemical effects were obtained in strong, and therefore probably homogeneous, fields.

It is not probable that the influence of the field on protoplasmic streaming is the reason for the inhibition of budding. This streaming in yeast is very slight and difficult to detect, so this possibility could not be tested.

SUMMARY

When old yeast cells are placed on the surface of a favorable solid medium, the magnetic field of a small horseshoe magnet retards the rate of bud formation by about 20 to 30 per cent.

Shields of glass or of paraffin placed between the magnet and the yeast do not interfere with the inhibitory action, but an iron shield prevents it. The non-magnetic end of the horseshoe magnet produces no effect.

A paraffin block, not wider than the poles, used as a shield deflects the lines of force sufficiently to change the curve of inhibition effects.

The yeast cells are sensitive to the action of the magnetic field only during the last half of the lag phase.

Five minutes exposure causes an inhibition; 10, 15, and 17 minutes produce no effect; 20, 25, 30, 60, and 150 minutes are inhibiting.

Any effect on the yeast buds is always associated with a heterogeneous field. Homogeneous fields produce no effect.

The most probable explanation is that some essential molecules in the cell are moved from their location, thus interrupting the normal progress of anabolism. Less probable seems the assumption that the magnetic field influences the rate of chemical reactions or of protoplasmic streaming.

The author wishes to acknowledge the valuable suggestions and criticisms offered by Prof. Otto Rahn, whose interest helped materially in carrying out this work.

REFERENCES

Bhatnagur, S. S., Nathur, R. N., and Kapur, R. N. 1929 The effects of a magnetic field on certain chemical reactions. Phil. Mag., 8, 457–473.

Chéveneau, C., and Bohn, G. 1903 De l'action du champ magnétique sur les Infusoires. C. R. Acad. Sc., 136, 1579–1580.

Ewart, A. 1903 Protoplasmic Streaming in Plants. Oxford.

Jennison, M. W. 1937 The growth of bacteria, yeasts, and molds in a strong magnetic field. Jour. Bact., 33, 15.

Kimball, G. C. 1937 The growth of yeast in a magnetic field. Thesis. Cornell University.

Leusden, F. P. 1929 Elektrische und magnetische Wirkung auf Bakterien. Centr. f. Bakt. (Abt. I), 111, 321–325.

Pissarschewsky, L. V. 1928 The reaction between metals and acids in a constant magnetic field. 2nd Phys. Chem. Conf., 24, 251–256. Quoted from Chem Abst., 26, 4217.

PISSARSCHEWSKY, L. V., AND ROSENBERG, M. A. 1923 Elektron in der Chemie der Lösungen und in der Elektrochemie. (Russian.) Quoted from Ssawostin (a).

RAHN, O. 1932 Physiology of Bacteria. Philadelphia, p. 263.

SSAWOSTIN, P. W. 1930a Magneto-physiologische Untersuchungen. I. Die Rotationsbewegung des Plasmas in einem konstanten magnetischen Kraftfelde. Planta, 11, 683–726.

SSAWOSTIN, P. W. 1930b Magnetwachstumsreaktionen bei Pflanzen. Planta, 12, 327–330.

TUTHILL, J. B., AND RAHN, O. 1933 Zum Nachweis mitogenetischer Strahlung durch Hefesprossung. Archiv f. Mikrobiol., 4, 565–573.

FURTHER STUDIES ON IV-VARIANTS OF SALMONELLA TYPHI-MURIUM (AERTRYCKE) WITH SPECIAL REFERENCE TO CULTURES FROM PIGEONS[1]

PHILIP R. EDWARDS

Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Kentucky

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Landsteiner and Levine (1932) were the first workers to note that certain cultures of *Salmonella typhi-murium* were devoid of antigen V of the Kauffmann-White schema. They noted this deficiency in the Binns strain of Schütze (1920). Kauffmann (1934) examined 256 cultures of *S. typhi-murium* and found 16 of them lacking in antigen V. The cultures exhibiting this peculiar deficiency in their somatic antigens, and designated by Kauffmann as *S. typhi-murium* var. *copenhagen,* are commonly referred to as IV-variants. Their antigenic formula is IV XII: i:1, 2, 3 instead of IV V XII : i : 1, 2, 3 which represents typical strains of *S. typhi-murium.* Zahn (1935) found one IV-variant in 74 cases of *S. typhi-murium* infection.

All the IV-variants described by the investigators cited above were isolated from human sources. Since it is well known that *S. typhi-murium* infection in man almost invariably has its origin in some animal reservoir of infection, it is important from an epidemiologic as well as an academic standpoint that the zoological distribution of the IV-variants be determined. Edwards (1935) reported that six cultures of *S. typhi-murium* isolated from pigeons belonged to the IV-variety. Hoffmann and Edwards (1937) reported the presence of IV-variants in an infection of rabbits. The rabbits were housed in hutches which

[1] The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

adjoined pens containing pigeons infected with the same organism. Except for these observations, there are no reports of the occurrence of the organisms in the lower animals. The purpose of the present paper is to present further observations on the occurrence of IV-variants in animals.

MATERIAL AND METHODS

The material to be reported consists of 155 cultures of *S. typhi-murium* of animal origin. The sources of the strains are as follows: horses, 14; sheep, 2; guinea pigs, 14; rats, 3; mice, 6; turkeys, 40; chickens, 10; ducks, 17; canaries, 11; pigeons, 35; rabbits, 3. These cultures have all been subjected to antigenic analysis. Particular attention has been paid to the somatic antigens in order to determine the presence or absence of factor V in the somatic complex. The flocculating antigens were examined by the method used by Edwards (1936). The somatic antigens were examined by the method of Kauffmann (1934).

RESULTS

It may be said at the outset that no IV-variants were found among the cultures from horses, sheep, guinea pigs, rats, mice, turkeys, chickens, ducks or canaries. Their occurrence was limited to the strains from pigeons and rabbits. On the contrary the 38 cultures from pigeons and rabbits were all IV-variants, no strains having the typical *S. typhi-murium* somatic complex being found among these cultures. Thirty-three cultures from pigeons were derived from 12 different flocks located in Connecticut, New York, New Jersey, South Carolina, Kentucky, Ohio, Colorado and California. Two cultures were of German origin. These were received from Dr. W. Herrmann of Essen.

It has been found by Kauffmann (1934) and Edwards (1935) that cultures of *S. typhi-murium* which lack factor V are likely to possess biochemical characteristics that are not typical of *S. typhi-murium*. Among the 38 cultures studied here there were 9 which failed to ferment maltose. Sixteen strains gave negative Bitter tests. Eight failed to utilize ammonium salts when tested

by the method of Hohn and Herrmann (1936). Four of the cultures were anaerogenic. The fermentation of maltose has no epizoological significance since maltose-positive and maltose-negative strains may be isolated from the same flock. Further, by cultivation in maltose broth and plating on Endo's agar containing maltose, it is possible to obtain maltose-fermenting sub-strains from cultures which fail to ferment maltose. Red daughter colonies appear on the plates. These ferment maltose rapidly. It has been found, however, that all strains from the same flock give identical results in the Bitter test. Also the 4 anaerogenic cultures comprised all the strains from one flock.

DISCUSSION

The IV-variants of *S. typhi-murium* form a definite entity. While they may be looked upon as a loss variant of typical cultures of the species, they are quite constant in their antigenic composition. Kauffmann (1934) noted no variation in the IV-variants he examined. The writer has maintained some of these strains as stock cultures for more than 3 years. During this time no changes have been noted in the antigenic characters of the bacilli. Emanuel (1936) was not able to induce any change in the somatic antigens although he used procedures that have induced antigenic variation in other genera.

Since *S. typhi-murium* is so widely distributed throughout the various species of domestic animals, it is surprising to find the zoological distribution of the variants so restricted. In the present study the organisms were found only in pigeons and rabbits. The rabbit strains are those reported by Hoffmann and Edwards (1937). Inasmuch as the rabbits were housed adjacent to pigeons carrying bacilli of identical antigenic and biochemical characters, these strains undoubtedly should be considered as originally of pigeon origin. The presence of these strains in rabbits demonstrates that the variants may be transferred from pigeons to other animal species.

Hohn and Herrmann (1936) and Herrmann (1936) have classified the *Salmonella* group according to the ability of the bacilli to utilize ammonium salts as a sole source of nitrogen. They

have advanced the theory that those cultures which fail to utilize ammonia are much more restricted in their host relationships than strains which are able to grow in a medium containing ammonium salts as the sole source of nitrogen. Among the organisms which fail to utilize ammonia are placed such organisms as *Eberthella typhosa* and *Salmonella paratyphi A* which ordinarily parasitize only man. On the other extreme are placed such ubiquitous species as *Salmonella typhi-murium* and the Newport type which readily utilize ammonia. Herrmann (1936a) states that IV-variants of *S. typhi-murium* which he has isolated from pigeons in Germany fail to grow on the synthetic medium of Hohn and Herrmann. The writer has confirmed Herrmann's observation, using the two German cultures available. The cultures isolated in the United States vary in their ability to utilize ammonia. Of the 36 American strain's studied, 28 grew readily on the medium of Hohn and Herrmann when either glucose or citrate was used as a source of carbon. The remaining 8 cultures failed to develop. The recent work of Kauffmann (1936) on single-colony isolations casts doubt on the value of ammonia utilization as a differential criterion.

It was found by Edwards (1936) that the cultures placed in the Binns type by White were antigenically identical with the pigeon cultures and with *S. typhi-murium* var. *copenhagen* of Kauffmann. Thus it is known that such variants are capable of producing disease in man. Likewise, it is well known that food poisoning may be caused by the consumption of pigeons or the eggs of pigeons infected with *S. typhi-murium*. The observations reported here demonstrate that the variants are enzootic in pigeons in the United States. The isolation of IV-variants from the human beings indicates that the infection was contracted either directly or indirectly from pigeons.

CONCLUSIONS

In a study of 155 cultures of *Salmonella typhi-murium* of animal origin it was found that the only IV-variants encountered were isolated from pigeons and from rabbits which had contact

with infected pigeons. Thirty-five cultures from pigeons all belonged to the IV-variety. This type is enzootic in pigeons in the United States. The occurrence of IV-variants of *Salmonella typhi-murium* in food poisoning indicates that the infection was contracted directly or indirectly from pigeons.

REFERENCES

EDWARDS, P. R. 1935 A serological variant of *Salmonella aertrycke* isolated from pigeons. Jour. Bact., 30, 465–471.

EDWARDS, P. R. 1936 Serological variants of *Salmonella typhi-murium* with special reference to *S. typhi-murium* var. *Binns*. Jour. Hyg., 36, 348–354.

EMANUEL, A. 1936 Variationsversuche an Bakterien der Paratyphusgruppe unter Berücksichtigung der Antigenfaktoren IV und V. Z. f. Immunitätsf., 87, 301–309.

HERMANN, W. 1936 Die akuten infektiösen Darmkrankheiten. Erg. d. ges. Med., 21, 239–274.

HERMANN, W. 1936a Personal communication.

HOFFMANN, H. A., AND EDWARDS, P. R. 1937 The spontaneous transmission of IV-variants of *Salmonella aertrycke* from pigeons to rabbits. Amer. Jour. Hyg., in press.

HOHN, J., AND HERRMANN, W. 1936 Der Kulturtyp der Erreger der Typhus-Paratyphus-Gruppe und seine Bedeutung für die Standortsgebundenheit. Z. f. Hyg., 117, 722–741.

KAUFFMANN, F. 1934 Über serologische und kulturelle Varianten der Paratyphus B- und Mäusetyphus-Bacillen. Z. f. Hyg., 116, 368–384.

KAUFFMANN F. 1936 Über die Bedeutung der Ammonnährboden für die Typentrennung in der Salmonella-Gruppe. Z. f. Hyg., 118, 425–428.

LANDSTEINER, K. AND LEVINE, P. 1932 On the Forssmann antigens in *B. paratyphosus B* and *B dysenteriae* Shiga. Jour. Immunol., 22, 75–82.

SCHUTZE, H. 1920 The paratyphoid B group. Lancet, i, 93–97.

ZAHN, E. 1935 Ueber das Vorkommen von "IV-Stämmen" bei Schottmüller- und Breslaubakterien. Z. f. Immunitätsf., 86, 162–164.

ADDENDUM

Since the above paper was prepared there has come to the attention of the writer a further paper by Hohn and Herrmann (Die Typen des Breslaubacteriums. Z. f. Hyg., 1937, 119, 369–382). In this paper the authors report the study of eleven strains of *S. typhi-murium* from pigeons. All these strains were IV variants. Three grew well in the medium of Hohn and Herrmann, while eight failed to grow. They conclude that failure

to utilize ammonium salts indicates that the organisms are of pigeon origin since they have not found this type in other animal species. They do not regard the lack of factor V as significant as they found one strain from a mouse and one strain from a calf which were also lacking in factor V. Nevertheless it seems significant that all of their pigeon strains were IV variants while only two strains from other species belonged to the variant type.

STUDIES OF FRESHWATER BACTERIA

IV. Seasonal Fluctuations of Lake Bacteria in Relation to Plankton Production[1]

ARTHUR T. HENRICI

Laboratories of Bacteriology at the University of Minnesota, Minneapolis, and at Station-on-Alexander, Cushing, Minnesota

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Inland lakes in the temperate zone undergo, through the year, a series of seasonal changes in physical and chemical characteristics dependent on changing temperature and light relationships. These changes in turn influence the quantity and kind of living organisms in the lake. Such changes in the life of inland lakes have been well studied so far as the larger organisms are concerned. But, although a number of studies of lake bacteria have been published, few of these have been carried on systematically over a period of time so that seasonal variations could be determined; and those papers which have reported on seasonal variations have been quite inconclusive.

Early studies were made at Lake Zürich by Kleiber (1894), Pfenniger (1902), and Minder (1920). Kleiber studied especially the fate of the large numbers of bacteria brought into the lake by inflowing streams. He noted that these had largely disappeared at a distance of 20 meters from the mouth. Pfenniger found that stream contamination affected the surface more than the deeper water. He reported the numbers of bacteria to be minimum during the summer stratification. The highest counts were obtained during the autumn following the death of plankton organisms. Minder confirmed the observation of Pfenniger, finding the highest bacterial counts in March and November, the lowest in midsummer. He believed that the summer mini-

[1] Aided by grants from the Graduate School of the University of Minnesota, and from the National Research Council.

mum was due to the lethal action of sunlight, and that food supply had little influence.

A summer minimum was also noted by Ruttner (1932) at Lake Lunz, and by Graham (1934) at Flathead Lake, with spring and autumn maxima. It is to be noted that all of these lakes are mountain lakes of the oligotrophic type. Ruttner believed that the turnover of the lake was responsible for the spring and fall maxima. Graham considered that autumn rains, and the melting of mountain snow in the spring, increased the bacteria by washing in material from the surrounding land.

The most extensive studies of seasonal fluctuations of lake bacteria have been made by Fred, Wilson and Davenport (1924) who reported observations on Lake Mendota, a stratified eutrophic lake of glacial origin in Wisconsin. Plate counts were made at regular intervals over a period of three years, and the results were found to vary considerably from year to year; there was a summer maximum in 1920, a fall maximum in 1921, and a spring maximum in 1922. They discuss the complexity of the factors affecting the numbers of bacteria in lake water, and emphasize the importance of rainfall. Lake Mendota is a drainage lake, and the bacterial counts are apparently considerably affected by the washing in of bacteria from the soil.

From this brief review of the scant literature it will be seen that such physical factors as sunlight, surface drainage, the stirring up of the water at the time of spring and fall turnover, and the summer stratification, have been considered more important in determining the seasonal fluctuations of lake bacteria than the production of organic matter by the plankton. It is true that the influence of the death of plankton in the water has been emphasized by Pfenniger (1902), Biega (1906) and Kolkwitz (1911), but their opinions are not well supported by the available data.

The observations here reported were made on a lake which does not stratify, and which was singularly free (during the period of observation) from surface drainage.

Lake Alexander is a hard-water, shallow, highly eutrophic lake, located in Morrison County near the center of Minnesota. It is

approximately $4\frac{1}{2}$ miles long and 2 miles across in the widest portion, with a maximum depth of 15 meters and an estimated mean depth of 9 meters. It is usually frozen from the middle of November to the middle of April, and does not stratify in the summer because it is so shallow and subject to strong wind action. It is a lake of the so-called "spring-fed" or seepage type, receiving drainage from a few small brooks only, none of which is more than 2 miles long. The observations here recorded were made during a year (1933) of very low rainfall. All of the inflowing brooks were completely dry throughout the year, and the lake level dropped about one meter. Consequently the washing of bacteria from the surrounding land into the lake was a negligible factor.

A float was anchored at the point marked *a* on the accompanying map (fig. 1). This point (Station 1) was chosen as being sufficiently far from shore to be truly representative of the open lake, yet convenient to the field laboratory. A second float, *b*, was anchored at a distance 50 meters from *a*, and a third one, *c*, at 300 meters. These were maintained in position throughout the period of observations, which were made at approximately weekly intervals from the time the ice went out in the spring until shortly before the freeze-up in the fall.

Six kinds of data were recorded, viz. the temperature of the water, the quantity of net plankton, the number of bacteria per cubic centimeter of water cultivable in an artificial medium (plate counts), the number of bacteria per square millimeter per day deposited on immersed glass slides (periphytic bacteria), the number of bacteria per cubic centimeter of the net plankton as determined by plate counts, and the number of bacteria per cubic centimeter of net plankton as determined by direct microscopic counts.

The temperature was determined by holding a laboratory thermometer about 0.5 meter below the surface until it came to equilibrium, and reading immediately.

The quantity of plankton was determined by towing a "Turtox" plankton net, conical in form, 24 cm. in diameter and 88 cm. long, of 20-mesh silk bolting cloth. This was towed for 300 meters (from *a* to *c*) when the plankton was scant, 50 meters

(from *a* to *b*) when it was abundant. The quantity of plankton has been recorded on the 50 meter basis, being divided by 6

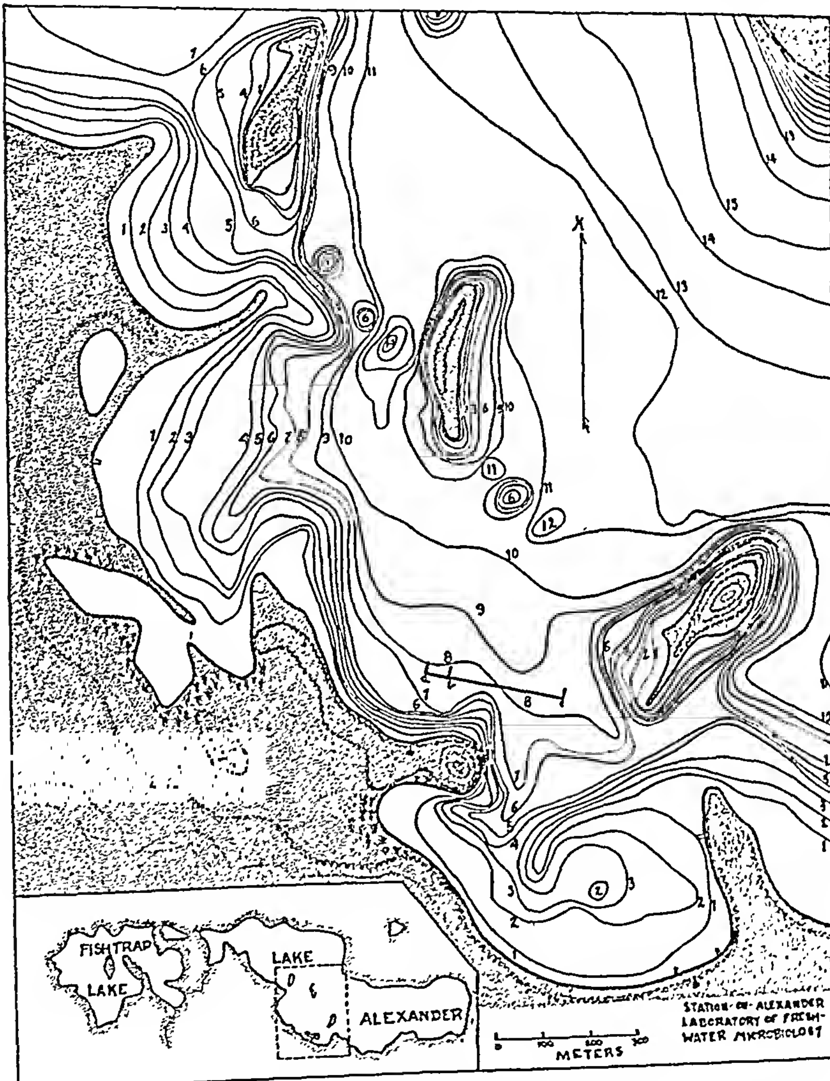

Fig. 1. Map of Lake Alexander, Showing Location of Station Where Data Were Collected

when the longer tows were made. Every effort was made to row the boat at a constant rate, so that the net, about 10 meters

behind, would sink to a depth of approximately 1 meter. The plankton was collected in vials attached to the apex of the net, and immediately transferred to a 4 per cent formaldehyde solution. On reaching the laboratory this was made up to a volume of 100 cc. and stored in a tightly stoppered bottle. At the end of the season all of the samples were transferred to 100 cc. graduated cylinders, also stoppered, and allowed to sediment for two weeks, at the end of which time the volume of plankton was read.

The method used for collecting and measuring the plankton is admittedly crude. The largest error is due to the fact that the different types of plankton organisms vary in the degree to which they form a compact sediment. The filamentous blue-green algae which formed the bulk of the plankton during the summer make a much less compact sediment, and therefore the relative quantities are somewhat exaggerated during this period. But the plankton measurements were made only for comparison with the bacteriological observations, and are, in the author's opinion, sufficiently accurate to show the trends.

Immediately after collecting the sample for measuring the plankton, the apex of the net was tied shut, and the net was towed over the same course. The net was held up to drain, and allowed to continue draining until the laboratory was reached, when the apical opening was untied, and samples of the compact net plankton were removed for bacteriological analysis.

A 5 cc. sample was removed by means of a 10 cc. graduated pipette with an opening approximately 4 mm. in diameter. This was transferred to a large bottle containing 95 cc. of sterile tap water and glass beads. After thorough shaking of this 1:20 dilution, further dilutions were made with 99 cc. water blanks, and quantitative plate cultures made by the same procedure used for plating the water samples.

With a 1 cc. tuberculin syringe, the tip of which had been cut off, another portion of the compact net plankton was removed, and 0.2 cc. was discharged upon the surface of a 50 x 75 mm. microscope slide. This slide had a line ruled across it with a wax pencil 25 mm. from one end, marking off an area 50 x 50 mm. A similarly marked slide was inverted over the first one, and the

compact plankton was "worked" over the measured areas, by rubbing one slide over the other, much as one prepares slides of sputum, continuing the "working" until the plankton was nearly dry. In this way the larger plankton organisms were crushed, and the entire mass spread in a fairly uniform film over the two slides. These were fixed by heat, stained with crystal violet, and the number of bacteria determined by counting 50 fields on each of the two slides. From these direct microscopic counts the number per cubic centimeter of the concentrated plankton was computed.

An examination of fresh plankton, either unstained or (better) mounted in Amann's fluid with cotton blue, reveals the presence of large numbers of bacteria. These are either embedded in the slime surrounding plankton algae, or on the chitinous surface of plankton animals, or in independent zoogloeal masses embedded in their own gum. In the smeared slides as described, the plankton organisms are largely disintegrated, or crushed so flat that the individual bacteria may be readily seen.

Samples of water for plating were collected in test-tubes which had been drawn out to a capillary tip, exhausted, sealed and sterilized. They were held under the water at a depth of about 0.5 meter, the tip broken by hand, and taken immediately to the field laboratory where they were plated. The plating medium used contains 0.05 per cent each of peptone, sodium caseinate, glycerol, starch and dibasic potassium phosphate, with 1.5 per cent agar, in tap water. This was put up in screw-cap lotion bottles of 120 cc. capacity, 30 cc. of agar to a bottle. These bottles were used in place of Petri dishes, the agar being melted and inoculated in the bottle, which was then placed on its side so that the agar hardened in a thin layer. The bottles were incubated with the caps tightly screwed, at room temperature, in the field laboratory. They were held for 1 to 3 weeks, depending on the temperature, before counting. The colonies may be readily counted through the glass wall of the bottle, using a hand lens. All samples were plated in 1 cc. quantities, undiluted and diluted 1:10, making 5 replicate plates from each dilution.

The periphytic bacteria were determined by suspending slides

from the under surface of the paraffined wooden float at the point *a* shown on the map. In all cases, two 50 x 75 mm. slides were used for each observation, and counts of 50 fields were made on each of the two slides. The technique of fastening, staining, and counting the slides has been described in previous publications, (Henrici, 1933, 1936). The middle portion of these slides was about 10 cm. below the water surface. Slides were immersed for varying periods, depending on the amount of growth, but in most cases for three weeks.

These data are presented in graphic form in figures 2 and 3. The total plankton, plate counts of water bacteria, and slide counts of periphytic bacteria are grouped together in figure 2; they seem to vary together in a significant manner. The counts of bacteria in the concentrated net plankton are presented in figure 3, together with the temperatures.

It will be seen from figure 2 that the plankton was produced in three distinct pulses, the first reaching its peak about June 1. During this period the dominant plankton organisms were diatoms (*Melosira*, *Fragilaria*, *Tabellaria*, and *Asterionella*). These decreased rapidly during June, reaching a minimum at July 1. During this period the plankton became quite heterogeneous, various green algae, and Protozoa (*Volvox*, *Ceratium*, and *Dinobryon*) becoming prominent. During July, when the water was constantly warm, the plankton again increased markedly, this increase being due almost entirely to blue-green algae (*Microcystis*, *Anabaena*, *Lyngbya*) which reached a maximum early in August when the lake was "blooming," i.e., on still days the entire surface of the water was covered with a greenish scum.

This pulse of blue-green algae decreased rapidly during August, the period of decrease being marked by a great increase of microscopic crustacea, which also disappeared rapidly. As the temperature of the water began to fall during the latter part of August, there was initiated a third plankton pulse that continued through September. The dominant organisms were again diatoms.

The plankton pulses of Lake Alexander differ somewhat from those which have been reported from other lakes. In most

cases, plankton studies have been made on deep, stratified lakes. These usually show two maxima, obviously associated with the spring and fall turnover of the stratified water which leads to general aeration and a distribution of food elements from the bottom deposit into the upper layers of water. Such deep lakes do not usually bloom during the summer. The very pronounced

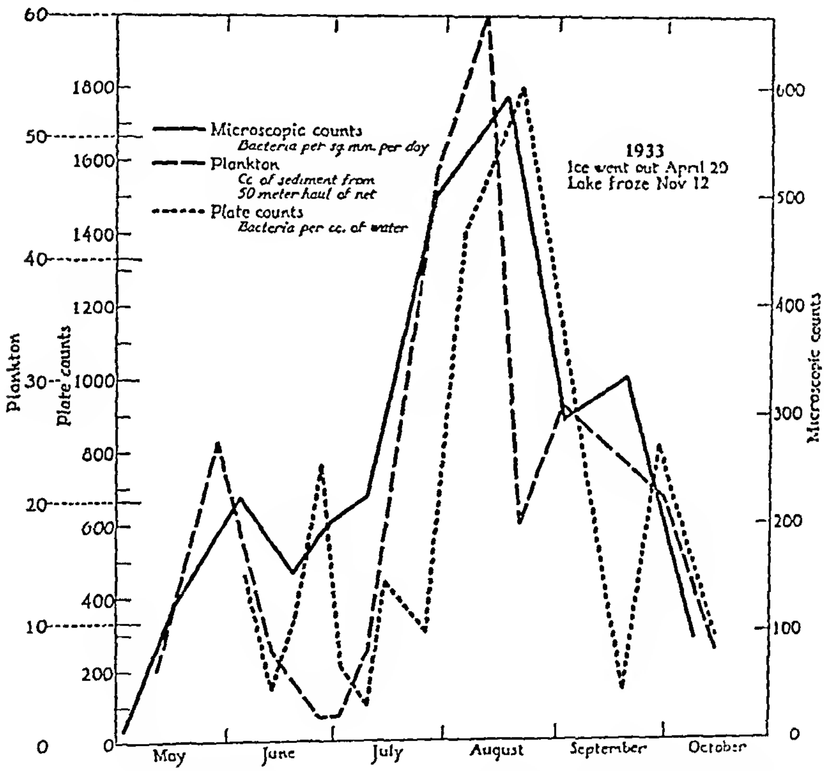

FIG. 2. SEASONAL FLUCTUATIONS OF TOTAL NET PLANKTON AND OF WATER BACTERIA IN LAKE ALEXANDER

midsummer plankton pulse of Lake Alexander may be due to the fact that the lake is not stratified, but is warmed and aerated to the bottom, and nutrient elements continuously circulated by wind action.

It will be seen from figure 2 that the curves for water bacteria also show three pulses during the year, which lag behind the

plankton pulse. The curve for the periphytic bacteria shows consistently a shorter lag than the curve for the plate counts of lake water. The amount of lag has also varied through the season. It must be remembered that the slides for the periphytic bacteria were immersed, on the average, for three weeks, the counts being plotted on the median date of the period of immersion. The curve does not, therefore, accurately indicate the time of greatest deposition of bacteria on the slides.

Since both curves for bacteria reproduce fairly well the curve for net plankton, these elements of life in the lake must be related, and the most obvious explanation of this relationship is that the plankton organisms provide organic matter which serves as food for the bacteria. Such a relationship has not been demonstrated in previous studies of lake bacteria because such studies have been made on lakes where the bacterial counts have been markedly affected by two other factors,—the semi-annual turnover which brings large numbers of bacteria from the bottom mud into the water, and the drainage of bacteria from the soil of the watershed into the lake.

The curves for bacteria in the concentrated net plankton are presented in figure 3. Since these represent bacteria per cubic centimeter of plankton, regardless of the volume of plankton in the lake water, it is not to be expected that they would follow the curves shown in figure 2. The bacteria trapped in the net plankton consist either of large zoogloeal colonies, or of smaller colonies attached to other plankton organisms. It was the author's impression, from microscopic studies of the wet plankton, that such bacteria were more abundant during midsummer when blue-green algae were dominant, but this impression is not supported by the actual counts. The very high peak in the curve for microscopic counts occurring at the beginning of June coincides with the dominance of the colonial flagellate, *Dinobryon*, in the plankton, the loricae and stalks of which were very heavily coated with bacteria. There is, almost throughout the series of observations, a curious negative correlation between the plate counts and the microscopic counts. This is probably accidental; at least the author can present no plausible explanation. Clumps

were very thoroughly broken up in preparing the smears, but probably not very well disintegrated by the shaking with beads preparatory to plating. While a study of the association of individual bacteria species with particular species of plankton

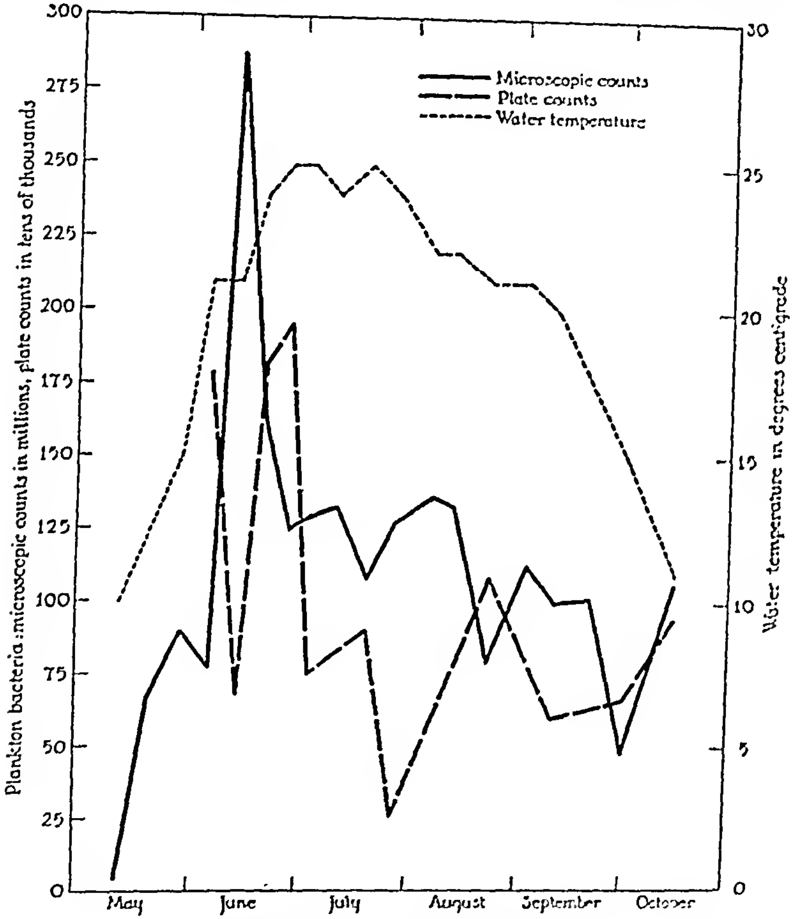

FIG. 3. SEASONAL FLUCTUATIONS OF WATER TEMPERATURE AND OF BACTERIA IN THE CONCENTRATED NET PLANKTON

organisms may prove fruitful, the general quantitative observations here reported do not seem to have yielded significant results.

SUMMARY

The quantity of total net plankton, plate counts of bacteria in the water, and microscopic counts of periphytic bacteria have

been determined in a shallow, unstratified lake of the seepage type, during a drought year when the lake received no surface drainage. Under these conditions it was found that the numbers of bacteria, as estimated by both methods, followed closely the curve for total plankton, with a lag which was greater in the case of the plate counts.

It is concluded that the production of organic matter by plankton organisms is an important factor in determining the number of bacteria in the water. Microscopic and plate counts of bacteria in the concentrated net plankton did not appear to fluctuate significantly through the seasons.

REFERENCES

Biega, M. 1906 Uber Wasserversorging aus dem Bodensee und die Beschaffenheit des Seewassers. J. f. Gasbel. u. Wasservers, 49. (Quoted by Baier: Arch. f. Mikrobiol., 1935, 29, 183-264).

Fred, E., Wilson, F., and Davenport, A. 1924 The distribution and significance of bacteria in Lake Mendota. Ecology, 5, 322-339.

Graham, V. 1934 A bacteriological study of Flathead Lake, Montana. Ecology, 15, 101-109.

Henrici, A. 1933 Studies of Freshwater Bacteria. I. A Direct Microscopic Method. Jour. Bact., 25, 277-286.

Henrici, A. 1936 III. Quantitative Aspects of the Direct Microscopic Method. Jour. Bact., 32, 265-280.

Kleiber, A. 1894 Qualitative und quantitative bakteriologische Untersuchungen des Zürichseewassers. Inaug. Diss. Zürich. (quoted by Fred, Wilson and Davenport).

Kolkwitz, R. 1911 Zur Biologie der Talsperren, insbesondere der Eschbachtalsperre bei Remscheid. Mitt. Prüf.-Anst. Wasservers. (quoted by Baier).

Minder, L. 1920 Zur Hydrophysik des Zürich- und Walensees, nebst Beitrag zur Hydrochemie und Hydrobakteriologie des Zürichsees. Arch. f. Hydrobiol., 12, 122-194.

Pfenniger, A. 1902 Beiträge zur Biologie des Zürichsees. Z. f. Gewässerkunde., 6, 321. (Quoted by Fred, Wilson and Davenport.)

Ruttner, F. 1932 Anhang zu "Beiträge zur Bakteriologie der Lunzer Seen," by A. Zih. Int. Rev. ges. Hydrobiol. und Hydrographie, 26, 431-438.

THE INFLUENCE OF VITAMIN C ON THE GROWTH OF ANAEROBES IN THE PRESENCE OF AIR, WITH SPECIAL REFERENCE TO THE RELATIVE SIGNIFICANCE OF EH AND O_2 IN THE GROWTH OF ANAEROBES

I. J. KLIGLER AND K. GUGGENHEIM[1]

Department of Hygiene and Bacteriology, Hebrew University, Jerusalem, Palestine

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INTRODUCTION

The problem of biotic activity in the absence of free oxygen has occupied the attention of physiologists and bacteriologists since the days of Pasteur. Pasteur (1861) postulated that free O_2 was lethal to anaerobic bacteria. This view has been contested by McLeod and Gordon (1923) who claimed that the toxic effect was due to peroxides; according to these authors the obligate anaerobes were devoid of catalase and hence could not destroy the peroxides formed by autooxidizable substances. This view was supported by Callow (1923) and received further support from the findings of Avery and Morgan (1924) that anaerobes grew freely under aerobic conditions if unheated, sterile, plant extracts were added to broth. The explanation advanced by these authors was that the plant catalases and peroxidases destroyed the peroxides formed and thus enabled the anaerobes to continue their growth.

This hypothesis was, however, rendered untenable by the findings of Novy (1925) and Sherman (1926). Novy showed that anaerobic growth occurred in one arm of an H tube if a piece of unsterilized potato or a culture of *Bacillus subtilis* was kept in the other arm; in other words, growth was possible when the free O_2 was removed from the tube. Sherman demonstrated that anaerobic propionic acid bacteria did produce catalase.

[1] F. M. Warburg Research Fellow.

And finally Quastel and Stephenson (1926) showed that H_2O_2 does not necessarily inhibit the growth of anaerobes under all conditions. These authors cultivated *Clostridium sporogenes* in the presence of small amounts of H_2O_2 in media containing 0.1 per cent cystein, glutathion or thioglycocol which lowered the reduction potential even in the presence of H_2O_2. Hosoya and Kishino (1925) obtained similar results with cystein broth (0.001 per cent cystein-hydrochloride) and Frei and Riedmueller (1930) with cystein-agar (1.0 per cent cystein in a 2.5 per cent agar).

More recently a number of investigators have pointed out the importance of the reduction potential in anaerobic growth. Coulter (1928), Dubos (1929), Aubel, Aubertin and Genevois (1929), Knight (1930), Plotz and Geloso (1930), Lepper and Martin (1930) have measured the reduction potential either by the potentiometer or by indicators. Plotz and Geloso (1930) have established that the minimum reduction potential for anaerobic growth is Eh +0.036 v. and Fildes (1929) and Fildes and Knight (1930) put it at Eh +0.11 v. Lepper and Martin showed that the Eh of the meat medium used for anaerobes is colorimetrically −0.2 v. and potentiometrically −0.174 v.

This view as to the importance of the reduction potential in the growth of anaerobes has been contested by Knaysi and Dutky (1934, 1936). On the basis of their investigations these authors concluded that the significant factor is the partial O_2 pressure, and that the reduction potential may merely serve as an indicator of the oxygen tension. They obtained good growth of *Clostridium butyricum* at low oxygen tension although the Eh was raised by the addition of potassium ferricyanide to +0.335 v.; on the other hand the same organism failed to grow when the O_2 tension was regulated so that the Eh of the medium was +0.3 v.

During the last two years we have been studying in this laboratory the effect of vitamin C on toxin production by *Corynebacterium diphtheriae* (Kligler 1936), as well as on viruses (Kligler and Bernkopf 1937). Since ascorbic acid belongs to the class of highly reducing substances and is at the same time

an important constituent of the plant and animal organism, we undertook a study of its effect on the growth of anaerobes.

In this paper we present the results obtained with *Clostridium welchii*.[2]

EXPERIMENTAL

I. The minimal amount of vitamin C required for "aerobic" growth of Clostridium welchii

The following medium was used in these experiments:

Meat extract	3.0 grams
Peptone	10.0 grams
NaCl	10.0 grams
K_2HPO_4	2.0 grams
H_2O	1000 grams
Reaction	pH 7.2–7.4

The medium was prepared in the usual manner, filtered, distributed in tubes and autoclaved. The vitamin C solution was made in distilled water and filtered through a Seitz filter. It was prepared fresh each time and desired amounts added to the broth just before inoculation with the culture. A 24-hour meat-broth culture was used for inoculation; 0.05 cc. of the liquid culture was added to each tube. The tubes were stoppered with cotton in the usual way.

Each experiment was repeated several times with the same results. We shall, therefore, present here only the results of type experiments.

The effect of various concentrations of vitamin C in broth on the growth of the culture is shown in table 1. It will be noted that growth occurred in the tubes containing 0.02 per cent but not in the tubes containing 0.01 per cent vitamin. When growth occurred, its intensity was the same in all tubes.

A similar series of experiments was made with semi-solid agar. This medium was prepared by the addition of 0.4 per cent agar to the stock broth used in the preceding experiment.

[2] An abstract of a somewhat similar investigation on the effect of vitamin C on anaerobic growth by Ehrisman (1936) came to our attention when our own experiments were concluded.

The results are shown in table 2. It will be noted that in this medium a much smaller amount of vitamin C sufficed for growth.

In plain broth the minimal amount of ascorbic acid for growth was 0.02 per cent while in the semi-solid agar it was 0.03 per thousand. It is also noteworthy that, whereas in the broth the turbidity is uniform in the entire tube, in the semi-solid agar growth stops short about 0.5 cm. from the top.

TABLE 1

Minimal concentration of vitamin C for growth of C. welchii in broth

CONCENTRATION OF VITAMIN C (PARTS PER THOUSAND)	DENSITY OF GROWTH (24 HOURS AT 37°C.)
2.0	++
1.0	++
0.5	++
0.2	++
0.1	–
0.05	–

TABLE 2

Minimal amount of vitamin C for growth of C. welchii in semi-solid agar

CONCENTRATION OF VITAMIN C (PARTS PER THOUSAND)	NATURE AND INTENSITY OF GROWTH
0.2	Upper 0.5 cm. sterile; lower part ++
0.1	Upper 0.5 cm. sterile; lower part ++
0.05	Upper 0.5 cm. sterile; lower part ++
0.03	Upper 0.5 cm. sterile; lower part ++
0.02	No growth
0.01	No growth

We next studied the effect of glucose. The media contained varying concentrations of glucose; the fluid media contained 1 part and the semi-solid media only 0.2 parts of vitamin C per thousand. The results are shown in table 3.

There was no growth in media containing 1.0 per cent glucose and only 0.1 per thousand vitamin.

It is apparent that glucose does not effect the concentration of vitamin C required for growth. It serves as a nutrient and hence better growth is obtained when it is present in sufficient amount.

Peptone proved to perform a different function. Simple media were prepared consisting of Tyrode solution with varying concentrations of peptone. The results are shown in table 4.

As can be seen from the table, no growth occurred in tubes

TABLE 3

The effect of glucose on the "aerobic" growth of C. welchii in media containing vitamin C

MEDIA	NATURE OF GROWTH
Broth + 1 per thousand vitamin C; + 0 glucose	++
Broth + 1 per thousand vitamin C; + 0.05 per cent glucose	++
Broth + 1 per thousand vitamin C; + 0.1 per cent glucose	++
Broth + 1 per thousand vitamin C; + 0.5 per cent glucose	+++
Broth + 1 per thousand vitamin C; + 1.0 per cent glucose	+++
Broth + 1.0 per cent glucose	−
Semi-solid + 0.2 per thousand vitamin C. + 0.0 per cent glucose	++*
Semi-solid + 0.2 per thousand vitamin C. + 0.5 per cent glucose	+++*
Semi-solid + 0.2 per thousand vitamin C. + 1.0 per cent glucose	+++*
Semi-solid + 1.0 per cent glucose	−†

* No growth 0.5 cm. from top.

† No growth.

TABLE 4

Effect of varying concentrations of peptone on the minimum concentration of vitamin C required for "aerobic" growth of C. welchii

CONCENTRATION OF VITAMIN C (PARTS PER THOUSAND)	CONCENTRATIONS OF PEPTONE (PER CENT)									
	0.0	0.25	0.5	1.0	1.5	2.0	2.5	3.0	4.0	5.0
1.0	−	±	+	+	++	++	++	++	+++	+++
0.2	−	−	±	+	+	++	++	++	++	+++
0.05	−	−	−	−	−	+	++	++	++	+++
0.02	−	−	−	−	−	−	−	−	−	−
0.00	−	−	−	−	−	−	−	−	−	−

−, ±, +, ++ = intensity of growth.

containing 0.02 per thousand vitamin C, no matter how high the concentration of peptone. However, above this amount there is a definite relation between the concentration of peptone and vitamin C; the greater the concentration of peptone, the less vitamin C is required. The intensity of growth also increases

with the concentration of peptone. Peptone serves, therefore, a double function; it provides reducing substances as well as nutrients.

II. *Fate of vitamin C in inoculated and uninoculated media*

It was naturally of interest to ascertain what happened to the vitamin C and in what way it exerted its influence on the cultures. Meat-extract broth containing varying concentrations of glucose and vitamin C was used. Parallel tubes, one inoculated and the other uninoculated, were incubated at 37° for 24 hours. At the end of this period the content of vitamin C was determined by titration with the Tillmans reagent. The results are shown in table 5.

TABLE 5

Loss of vitamin C in inoculated and uninoculated broth containing glucose

CONCENTRATION OF GLUCOSE	INOCULATED	GROWTH	AMOUNT OF VITAMIN C	LOSS OF VITAMIN C	
(a) 5 mgm. vitamin C per 5 cc. medium = 1 per thousand; findings after 21 hours					
per cent			*mgm.*	*mgm.*	*per cent*
0.0	−	−	2.8	2.2	44.0
0.0	+	++	3.6	1.4	28.0
0.05	−	−	2.8	2.2	44.0
0.05	+	++	3.2	1.8	36.0
0.10	−	−	2.8	2.2	44.0
0.10	+	++	3.6	1.4	28.0
0.50	−	−	2.7	2.3	46.0
0.50	+	+++	3.4	1.6	32.0
(b) 10 mgm. vitamin C per 5 cc. = 2 per thousand					
0.0	−	−	7.1	2.9	29.0
0.0	+	++	8.4	1.6	16.0
0.05	−	−	7.2	2.8	28.0
0.05	+	++	8.4	1.6	16.0
0.10	−	−	7.2	2.8	28.0
0.10	+	++	8.7	1.3	13.0
0.50	−	−	7.3	2.7	27.0
0.50	+	+++	8.6	1.4	14.0

It is interesting to note that the actual loss in the amount of vitamin C is the same in media containing 2:1000 as in those

containing 1:1000 of vitamin. The actual loss in milligrams in 5 cc. was 2.2 to 2.9. Whether this loss is due to oxidation or to combination with substances in the media is now under investigation (Leibowitz and Guggenheim). Another point of interest is the fact that the loss of vitamin was about twice as high in the uninoculated as in the inoculated tubes. It is evident that bacterial growth inhibits the loss, possibly as a result of the reducing substances produced by the bacteria. Glucose exerts no influence on the vitamin either in the inoculated or uninoculated tubes.

TABLE 6

Influence of peptone on the loss of vitamin C in media containing

CONCENTRATION OF PEPTONE	INOCULATED	GROWTH	AMOUNT OF VITAMIN C	LOSS OF VITAMIN C	
(a) 5 mgm. vitamin per 5 cc. = 1 per thousand; findings after 24 hours					
per cent			*mgm.*	*mgm.*	*per cent*
0.0	Control	−	1.25	3.75	75.0
0.5	−	−	2.1	2.9	58.0
0.5	+	±	2.3	2.7	54.0
1.0	−	−	2.5	2.5	50.0
1.0	+	+	2.5	2.5	50.0
2.0	−	−	2.6	2.4	48.0
2.0	+	++	2.6	2.4	48.0
4.0	−	−	2.7	2.3	46.0
4.0	+	+++	2.7	2.3	46.0
(b) 10 mgm. vitamin C per 5 cc. = 2 per thousand					
0.0	Control	−	4.8	5.2	52.0
0.5	−	−	5.1	4.9	49.0
0.5	+	±	5.3	4.7	47.0
1.0	−	−	6.9	3.1	31.0
1.0	+	+	7.0	3.0	30.0
2.0	−	−	7.1	2.9	29.0
2.0	+	++	7.1	2.9	29.0
4.0	−	−	7.0	3.0	30.0
4.0	+	+++	7.1	2.9	29.0

Similar analyses were made of media containing peptone. These media consisted of Tyrode solution to which were added various concentrations of vitamin and peptone respectively. The results are shown in table 6.

These results differ from those obtained in the glucose media. Whereas glucose has no effect on the vitamin, peptone exercises a protective influence on vitamin C. The loss of the vitamin in the media decreases with increasing amounts of peptone up to 1 per cent; beyond that point no difference is noted. However, in contrast with the glucose-broth, the growth of bacteria in the *tyrode-peptone* media does not modify the rate of loss of vitamin C. There is apparently a limiting protective or sparing effect and in peptone media this is exerted by the peptone. It is also evident that the bacteria do not use vitamin C as a nutriment.

These results complement those reported earlier in this paper on the influence of glucose and peptone on the vitamin C minimum for growth. Glucose which, as shown by subsequent experiments, does not influence the reduction potential, also fails to prevent oxidation of vitamin C; it improves growth only when a sufficient amount of vitamin C is present to make growth possible. Peptone, on the other hand, which lowers the reduction potential, protects vitamin C and permits growth, within certain limits, even in the presence of smaller concentrations of vitamin C. The vitamin obviously serves the function of a catalyzer.

III. Determination of reduction potential

Having established the conditions favouring the growth of anaerobes in the presence of air, we proceeded to determine whether the reduction potential or the oxygen concentration was the determining factor in the growth of anaerobes. The measurements of the reduction potential were made colorimetrically with indigo-carmin. To every 5 cc. of the medium we added 0.1 cc. of a 0.5 per cent solution of the indicator. The media were not freed of air, since we were concerned with the reduction potential under conditions under which growth occurred. The color change which occurred during 24 hours was determined in the usual way in a colorimeter.

The results are given in tables 7 and 8. It is apparent that the intensity of reduction parallels the increasing concentrations

of vitamin. Glucose does not influence the reduction. It is also of interest to note that the minimum amount of vitamin making growth possible, i.e., 0.2 per thousand causes a reduction of about 75 per cent, equivalent to an Eh −0.125 v. Growth does not occur when the amount is such that the reduction of indigo-carmin is less than 75 per cent.

TABLE 7

Reduction of indigo-carmin in media containing various concentrations of vitamin, with and without glucose (Basic medium beef-extract broth)

CONCENTRATION OF VITAMIN	CONCENTRATION OF GLUCOSE	REDUCTION OF INDICATOR	GROWTH
parts per thousand	*per cent*	*per cent*	
2.0	0.0	100	+
1.0	0.0	90	+
0.5	0.0	75	+
0.1	0.0	50	−
1.0	0.5	80	+
0.5	0.5	75	+
0.1	0.5	50	−

TABLE 8

Reduction of indigo-carmin in media containing various concentrations of vitamin C and peptone in Tyrode solution

CONCENTRATION OF VITAMIN	CONCENTRATION OF PEPTONE (PER CENT)								
	0	0.25	0.5	1.0	1.5	2.0	3.0	4.0	5.0
parts per thousand									
1.0	0*	75	90	90	90	90	90	100	100
0.2	0	50	75	75	75	90	90	90	90
0.05	0	25	50	50	50	75	75	75	75

* The figures represent per cent reduction. It should be noted that there is a limiting point where no increased reduction is obtained no matter how much peptone is added.

As was to be expected from the preceding experiments, peptone itself exerts a reducing effect. With increasing amounts of peptone, smaller quantities of Vitamin C are sufficient to give 75 per cent or more reduction of the indicator.

If table 8 is compared with table 4, it becomes apparent that

there is a close parallelism between the intensity of reduction and the ability of the organisms to grow. Growth occurred in all mixtures of vitamin-peptone which gave 75 per cent reduction of the indicator or an Eh of -0.125 v. Wherever the reduction was less, no growth occurred.

IV. The oxygen content of the Vitamin-C-containing media

In order to establish whether the free O_2 or the reduction potential was the determining factor in the growth of anaerobes, it

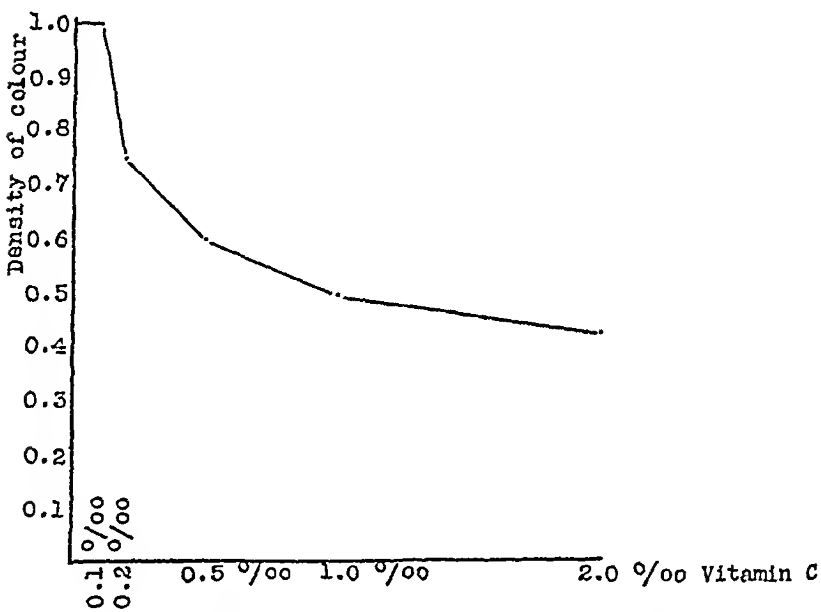

Fig. 1. Effect of Vitamin C on O_2 Content of Broth

was essential to ascertain whether, in addition to lowering the reduction potential, the vitamin also reduced the oxygen content of the medium by autooxidation. Pyrogallol was used to measure the oxygen in the medium. To every 5 cc. of broth, containing varying quantities of vitamin C, we added 0.1 cc. of a 2 per cent aqueous solution of pyrogallol and 0.1 cc. of a 5 per cent solution of NaOH; after 15 to 20 minutes the intensity of color was measured in a colorimeter, broth without vitamin C serving as the standard. Taking the color of the broth as 1.0,

it was possible to grade the shades of color in the tubes containing various concentrations of vitamin. The results are given in table 9 and figure 1.

It will be noted that considerable amounts of free oxygen were still present in the tubes in which growth occurred. In other words, the amount of vitamin C sufficient to make growth possible used up only a relatively small amount of the free oxygen.

In order to obtain some idea of the oxygen tension in the tubes supporting growth, the pyrogallol test described above was applied to broth under different oxygen tensions. The broth tubes were fitted with two-holed rubber stoppers containing glass capillaries and evacuated to varying degrees. After the evacuation 0.1 cc. pyrogallol and 0.1 cc. NaOH were sucked into the tubes and the intensity of color measured in the manner described above. It was found that an air pressure of 675 mm. or a partial oxygen pressure of 135 mm. gave a color with pyrogallol equal to 0.71 of the standard. The relation of color density of the pyrogallol to O_2 tension is shown in figure 2. It will be noted that a reduction of only 3 mm. in the oxygen tension gives a color reaction at which growth occurred in the presence of vitamin C.

TABLE 9

The relative amount of free oxygen found in broth containing different amounts of vitamin C

CONCENTRATION OF VITAMIN (PARTS PER THOUSAND)	RELATIVE INTENSITY OF COLOR	GROWTH OF ANAEROBE, (C. WELCHII)
0.0 (control)	1.0	–
0.05	1.0	–
0.1	1.0	–
0.2	0.75	+
0.5	0.6	+
1.0	0.5	+
2.0	0.43	+

It is apparent, therefore, that growth of *Clostridium welchii* occurred in tubes with over 95 per cent of the normal oxygen tension, provided an adequate amount of vitamin C was present.

In other words, the significant function of vitamin C is to lower the reduction potential.

These results were confirmed in another way. Broth tubes containing varying amounts of vitamin C were stoppered with corks and left in the incubator overnight. The next day they were examined by the pyragallol method described above. All tubes gave a color density of 0.9 to 1.0. Apparently, during

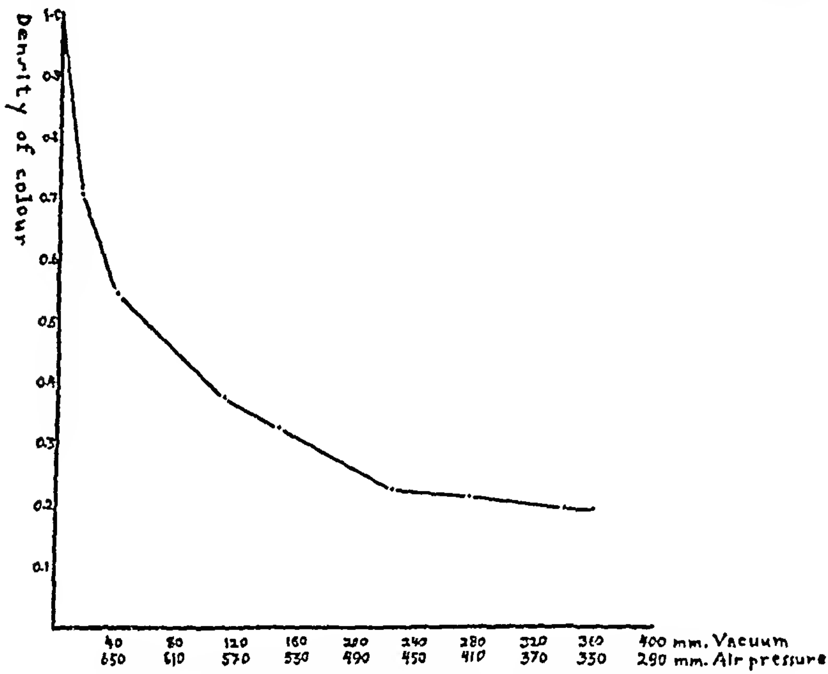

FIG. 2. RELATION OF O_2 CONCENTRATION TO COLOR DENSITY OF PYROGALLOL SOLUTION

the night the oxygen used up by the vitamin is replaced from the air in the tube, so that practically "aerobic" conditions existed even in tubes containing 2 parts per thousand of the vitamin.

Further evidence that the reduction potential was the decisive factor in the growth of *Clostridium welchii* under "aerobic" conditions was furnished by another type of experiment. In this experiment we added to all tubes 0.05 per thousand vitamin

C, an amount inadequate for growth in broth. The media were inoculated with *Clostridium welchii* and then the tubes were evacuated in varying degrees, the maximum being 315 mm. No growth occurred in any of the tubes although the pyrogallol test in the last tubes was 0.2 of the standard. It appears then that when the reduction potential was not suitable, no growth occurred, even when the O_2 tension was far below that found in tubes where good growth occurred.

DISCUSSION

The experiments reported above appear to us to clear up the moot point as to the determining factor in the growth of anaerobes—if *Clostridium welchii* is accepted as a type. It seems that anaerobiosis is determined by the reduction potential of the medium rather than by other factors. The favorable effect of plant juices is presumably due to the reducing substances present and not to the catalase and peroxidase as postulated by Avery and Morgan (1924).

It is not easy to establish the mechanism involved. But it may be assumed that the enzymic or rather catalytic system of anaerobes can only be active at a given reduction potential. At that potential, free O_2 does not interfere with its oxidation-reduction activity. At a point above that potential, the free oxygen interferes with the function of the anaerobes and growth is not possible. An illustrative experiment in this connection is reported by Wurmser (1925). This author showed that the synthesis of alanin from pyruvic acid and ammonia with glucose as H donator is possible only at an Eh less than 22; at a higher Eh the synthesis does not occur, the glucose reacts with the free oxygen, is oxidized and fails to act as an H donator.

This view enables us also to explain the results of Knaysi and Dutky, who obtained growth of anaerobes at a high reduction potential in the absence of oxygen. So long as oxygen is absent, there can be no interference with the cell oxidation-reduction processes. In the presence of O_2, however, these processes are possible only when the reduction potential is so low that O_2 interference is eliminated.

These experiments also suggest the function of Vitamin C in the organism. On the one hand, it serves as a catalyzer and makes possible reactions which are dependent on its powerful reducing properties. On the other hand, it is dependent on other reducing substances to prevent its own oxidation. It is this constant oxidation which necessitates the continual replacement of vitamin C.

SUMMARY

1. Addition of vitamin C to fluid media favors the growth of anaerobes (*Clostridium welchii*) in the presence of air.

2. Glucose improves growth but does not affect the minimal amount of vitamin required to make growth possible.

3. In tyrode-peptone media the amount of vitamin C required to make growth possible decreases (within limits) as the concentration of peptone is increased. If the concentration of vitamin C is below 0.05 per thousand, growth fails even if large amounts of peptone are added.

4. The reduction potential of the medium at which growth of *Clostridium welchii* occurred was about −0.125 v. The addition of glucose does not modify the potential and exerts no influence on the loss of vitamin C. The addition of peptone lowers the potential and exerts a protective effect on the vitamin added to the medium.

5. Vitamin C presumably acts as a catalyzer for anaerobes. It lowers the reduction potential of the media but does not appreciably affect the O_2 tension.

6. These experiments indicate that anaerobic growth is determined by the Eh and not by O_2 tension. Growth is possible in the presence of air when the Eh is sufficiently low. If the Eh is above the critical point, free O_2 interferes with the oxidation-reduction processes of the cell.

REFERENCES

Aubel, R., Aubertin, E., and Génevois, L. 1929 Sur le potentiel d'oxydo-réduction de la levure, des anaérobies facultatifs, des anaérobies strict-, et des milieux où vivent ces organismes. Ann. Physiol. et Physico-chim. Biol., 5, 1–10.

AVERY, O. T., AND MORGAN, H. J. 1924 Studies on bacterial nutrition. V. The effect of plant tissue upon the growth of anaerobic bacilli. Jour. Exp. Med., 39, 289-302.

CALLOW, A. B. 1923 On catalse in bacteria and its relation to anaerobiosis. Jour. Path. and Bact., 26, 320-325.

COULTER, C. B. 1928 Oxidation-reduction equilibria in biological systems. I. Reduction potentials in sterile culture bullion. Jour. Gen. Physiol., 12, 139-146.

DUBOS, R. 1929 The initiation of growth of certain facultative anaerobes as related to oxidation-reduction process in the medium. Jour. Exp. Med., 49, 559-592.

EHRISMANN, O. 1936 Askorbinsäurehaltige Nährmedien für anaerobe Bacillen. Zeitschr. fur Hyg., 118, 544-554; Bull. Hyg., 12, 62.

FILDES, P. 1929 The positive limits of oxidation-reduction potential required for the germination of spores of *B. tetani* in vitro. Brit. Jour. Path. and Bact., 10, 151-175.

FILDES, P., AND KNIGHT, B. C. J. C. 1930 Oxidation-reduction studies in relation to bacterial growth. III. The positive limits of oxidation-reduction potential required for the germination of *B. tetani* spores in vitro. Biochem. Jour., 24, 1496-1502.

FREI, W., AND RIEDMUELLER, L. 1930 Die Verwendung des Zysteins bei der Anaerobenzüchtung. Zentr. Bakt., I, Orig., 119, 282-288.

HOSOYA, A., AND KISHINO, S. 1925 Scient. Rep. Gov. Inst. Inf. Dis., Tokyo, 4, 103-123.

KLIGLER, I. J. 1936 Inhibitive effect of vitamin C on toxin production by *C. diphtheria*. Nature, 138, 291.

KLIGLER, I. J., AND BERNKOPF, H. 1937 Inactivation of vaccine virus by ascorbic acid and glutathione. Nature, 139, 965-966.

KNAYSI, G., AND DUTKY, S. R. 1934 The growth of *B. megatherium* in relation to oxidation-reduction potential and the oxygen content of the medium. Jour. Bact., 27, 109-119.

KNAYSI, G., AND DUTKY, S. R. 1936 The growth of butanol *Clostridium* in relation to the oxidation-reduction potential and the oxygen content of the medium. Jour. Bact., 31, 137-149.

KNIGHT, B. C. J. C. 1930 Oxidation-reduction studies in relation to bacterial growth. I. The oxidation-reduction potential of sterile meat broth. Biochem. Jour., 24, 1066-1079.

LEIBOWITZ, J., AND GUGGENHEIM, K. Unpublished results.

LEPPER, E., AND MARTIN, C. J. 1930 Oxidation-reduction potential of cooked meat media. Brit. Jour. Exp. Path., 11, 137-139.

McLEOD, J. W., AND GORDON, J. 1923 Catalase production and sensitiveness to hydrogen peroxide amongst bacteri: with a scheme of classification based upon these properties. Jour. Path and Bact., 26, 326-331.

McLEOD, J. W., AND GORDON, J. 1923 The problem of intolerance to oxygen by anaerobic bacteria. Jour. Path. and Bact., 26, 332-343.

NOVY, F. G., Jr. 1925 Microbic respiration. IV. The so-called aerobic growth of anaerobes: potato respiration. Jour. Inf. Dis., 36, 343-382.

PASTEUR, L. 1861 Animalcules infusoires vivants sans gaz oxygène libre et determinant des fermentations. Compt. rend. Acad. Sc., 52, 344-347.

PLOTZ, H., AND GELOSO, J. 1930 Relations entre la croissance des microorganismes anaérobies et le potentiel du milieu de culture. Ann. Inst. Pasteur, 43, 613-640.

QUASTEL, J. H., AND STEPHENSON, M. 1926 Experiments on "strict" anaerobes. I. The relationship of *B. sporogenes* to oxygen. Bioch. Jour., 20, 1125-1137.

SHERMAN, J. M. 1926 The production of catalase by an anaerobic organism. Jour. Bact., 11, 417-418.

WURMSER, R. 1925 Sur le potentiel d'oxydation cellulaire et les phénomenes d'oxydo-réduction. Compt. rend. Soc. Biol., 93, 1478-1479.

THE FERMENTATION OF ACETYL-METHYL-CARBINOL BY THE ESCHERICHIA-AEROBACTER GROUP AND ITS SIGNIFICANCE IN THE VOGES-PROSKAUER REACTION[1]

RALPH P. TITTSLER

Department of Bacteriology, University of Rochester, School of Medicine and Dentistry, Rochester, New York

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The ability of certain members of the *Aerobacter* genus to ferment acetyl-methyl-carbinol is indicated by their transitory Voges-Proskauer reactions. These strains produce acetyl-methyl-carbinol from glucose and, consequently, yield positive Voges-Proskauer reactions after incubation for from one to three days in Clark and Lubs' medium (1915). When incubation is continued for longer periods, however, the reactions become negative. Paine (1927), and Williams and Morrow (1928) showed that these bacteria "destroyed" the acetyl-methyl-carbinol which was present in filtrates of young cultures. In their experiments, the sterile filtrates were inoculated with the cultures to be tested and, after various periods of incubation, the presence or absence of acetyl-methyl-carbinol was determined by the Voges-Proskauer method. A negative reaction was considered as evidence that the acetyl-methyl-carbinol had been "destroyed." Supplementary experiments showed that the negative reactions were not due to an exhaustion of peptone. Williams and Morrow concluded, therefore, that "it seems probable that the compound serves as a source of carbon."

Although the work just cited showed that acetyl-methyl-carbinol is "destroyed" by certain members of the *Escherichia*-

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Aerobacter group, it was not clear whether the bacteria fermented it or reduced it to 2,3-butylene glycol.

In view of the importance given to the Voges-Proskauer reaction in the separation of the *Escherichia* and *Aerobacter* genera, and because of the relationship between the content of acetyl-methyl-carbinol and the flavor of certain foods, it seemed to be important to determine directly the ability of *Escherichia-Aerobacter* strains to ferment acetyl-methyl-carbinol and to utilize this compound as the sole source of carbon. The present paper gives the results of such an investigation.

MATERIALS AND METHODS

In all, 175 cultures, representing all of the common species of the *Escherichia-Aerobacter* genera, were employed. Of these strains, 130 had been isolated recently from water or from human feces and urine. The others were selected from the laboratory collection. The characteristics of each culture were determined in detail to permit exact taxonomic allocations to be made.

Fermentation tests were carried out in a liquid medium made of peptone, 0.5 per cent; meat extract, 0.3 per cent and acetyl-methyl-carbinol (Lucidol or Eastman), 1 per cent, and adjusted to pH 6.8 or 7.0. Either bromcresol purple or bromthymol blue was added to serve as an indicator. The ability of the bacteria to utilize acetyl-methyl-carbinol, when present in the medium as the sole source of carbon, was determined in a synthetic medium composed of $Na(NH_4)HPO_4 + 4H_2O$, 1.5 grams; KH_2PO_4, 1 gram; $MgSO_4$, 0.2 gram; $CH_3 \cdot CO \cdot CHOH \cdot CH_3$, 2 grams and distilled water, 1000 cc. This medium is a modification of Koser's (1924) citrate medium. In certain experiments the amount of acetyl-methyl-carbinol in the synthetic medium was varied so that it contained 0.5, 1, or 4 grams. The pH of this medium was also 6.8 or 7.0. The media were dispensed in chemically clean culture tubes. Most lots of media were sterilized by filtration through a Berkefeld N candle, but some were sterilized in the autoclave at a pressure of 15 pounds for 20 minutes. Inoculations were made with a needle from broth cultures that were from 12 to 18 hours old. Incubation was at

37°C. The results of the fermentation tests in nutrient broth were recorded daily for 14 days. The synthetic medium was examined daily for 6 days to detect growth. Every strain which grew in the synthetic medium was carried through at least 2 serial passages to determine its continued ability to utilize acetyl-methyl-carbinol as a sole source of carbon. At the completion of the incubation period, the reaction of the synthetic medium was determined by the addition of a few drops of bromthymol blue.

RESULTS

Acetyl-methyl-carbinol was fermented by 62 per cent of the strains of *Aerobacter oxytocum* and by 50 per cent of the strains of *Aerobacter aerogenes*, but not by any of the strains of *Aerobacter cloacae*, *Aerobacter levans*, or by any of the members of either the *Escherichia* genus or the *Escherichia-Aerobacter* "intermediate" group. There was never any indication of gas production. Every strain belonging to the *Aerobacter* genus, with the exception of two, which fermented acetyl-methyl-carbinol in nutrient broth also grew and produced acid in the synthetic medium, and gave a negative Voges-Proskauer reaction in Clark and Lubs' medium, when incubation was continued for from 3 to 5 days. The results of the various tests are summarized in table 1.

Usually, fermentation in nutrient broth was evident within from 2 to 4 days, but with some cultures it could not be detected before from 5 to 8 days. In general, growth occurred in the synthetic medium within 24 hours, but with the strains which exhibited delayed fermentation in nutrient broth it was not evident until the second or third day. The rapidity of fermentation in broth and the growth in the synthetic medium paralleled the rate of destruction of acetyl-methyl-carbinol during continued incubation in Clark and Lubs' medium.

The media which were sterilized in the autoclave and those sterilized by filtration yielded identical results.

In general, the variations in the content of acetyl-methyl-carbinol in the synthetic medium did not affect either the rapidity or the amount of bacterial growth. With a few strains,

however, there was better growth in the media which contained either 0.2 or 0.4 per cent of acetyl-methyl-carbinol than in those which contained either 0.05 or 0.1 per cent.

Bromcresol purple and bromthymol blue served equally well to indicate fermentation.

No correlation was found between the ability to ferment acetyl-methyl-carbinol and any other cultural characteristic.

TABLE 1

Relationship between the fermentation of acetyl-methyl-carbinol and the Voges-Proskauer reaction

SPECIES	NUMBER OF STRAINS	FERMENTATION OF ACETYL-METHYL-CARBINOL		VOGES-PROSKAUER REACTION (CLARK AND LUBS' MEDIUM) AFTER INCUBATION FOR	
		Nutrient broth	Synthetic medium	1 to 3 days	5 days
Aerobacter aerogenes	15	+	+	+	−
	2	+	−	+	−
	17	−	−	+	+
Aerobacter oxytocum	20	+	+	+	−
	12	−	−	+	+
Aerobacter cloacae	22	−	−	+	+
Aerobacter levans	2	−	−	+	+
Escherichia group	50	−	−	−	−
Escherichia-Aerobacter "intermediate" group	35	−	−	−	−

DISCUSSION

The results show that within the *Escherichia-Aerobacter* group the ability to ferment acetyl-methyl-carbinol is limited to certain strains of *Aerobacter aerogenes* and *Aerobacter oxytocum*, and that only these particular members of the *Aerobacter* genus yield negative Voges-Proskauer reactions in Clark and Lubs' medium when the cultures are incubated for periods exceeding 3 to 5 days. This finding does not interfere with the usefulness of the Voges-Proskauer reaction as a means of separating members of the *Escherichia* and *Aerobacter* genera. It simply necessitates the use of cultures which are less than 3 days old. This is a shorter period of incubation than is usually recommended for

routine tests, for example, a period of 4 days is recommended in Standard Methods of Water Analysis (A. P. H. A., 1933). When O'Meara's modification of the Voges-Proskauer test and either Clark and Lubs' medium or Difco V-P medium are employed, almost every member of the *Aerobacter* genus will yield a positive reaction after incubation for from 12 to 48 hours (Tittsler, 1933; and Levine *et al.*, 1934). Accordingly, cultures to be tested for the production of acetyl-methyl-carbinol should not be more than 2 days old.

All strains of *Aerobacter aerogenes* and *Aerobacter oxytocum* produce acetyl-methyl-carbinol from glucose and certain other carbohydrates, but only certain strains are able to ferment it. Obviously, therefore, the intermediate metabolism of all strains is not the same. These bacteria also differ from the citric-acid-fermenting streptococci which reduce acetyl-methyl-carbinol to 2,3-butylene glycol (Hammer *et al.*, 1935). Furthermore, Werkman (1930) found that various cultures of the *Aerobacter* genus produce acetyl-methyl-carbinol from 2,3-butylene glycol. There is evidence which suggests that these various activities are related to differences in oxidation-reduction potentials.

On the basis of this study it cannot be stated whether or not some members of the *Aerobacter* genus will ferment the acetyl-methyl-carbinol in certain foods such as butter, bread, coffee and honey, or in tobacco and beer. It is probable, however, that they may under suitable environmental conditions. If they do, the desirable aroma and flavor of many foods will be affected adversely, because these characteristics are dependent upon diacetyl, an oxidation product of acetyl-methyl-carbinol (van Niel, Kluyver and Derx, 1929; Schmalfuss and Barthmeyer, 1929; Visser't Hooft and de Leeuw, 1935; and Hammer, 1935).

SUMMARY

1. The ability to ferment acetyl-methyl-carbinol has been determined for 175 strains of the *Escherichia-Aerobacter* group.

2. Approximately one-half of the strains of *Aerobacter aerogenes* and *Aerobacter oxytocum* fermented this substance, but it was not attacked by any of the strains of *Aerobacter cloacae*, *Aero-*

bacter levans, or by any of the members of either the *Escherichia* genus or the *Escherichia-Aerobacter* "intermediate" group.

3. Every strain which fermented acetyl-methyl-carbinol also grew and produced acid in a synthetic medium which contained acetyl-methyl-carbinol as the sole source of carbon. These strains also gave negative Voges-Proskauer reactions in Clark and Lubs' medium, when the cultures were incubated for periods exceeding from 3 to 5 days.

4. Cultures of the *Escherichia-Aerobacter* group to be tested for the production of acetyl-methyl-carbinol should not be more than 2 days old.

5. The relation of acetyl-methyl-carbinol to the flavor of certain foods is discussed.

REFERENCES

CLARK, W. M., AND LUBS, H. A. 1915 The differentiation of the colon-aerogenes family by the use of indicators. Jour. Inf. Dis., 17, 160–173.

HAMMER, B. W., STAHLY, G. L., WERKMAN, C. H., AND MICHAELIAN, M. B. 1935 Reduction of acetylmethylcarbinol and diacetyl to 2,3-butylene glycol by the citric acid fermenting streptococci of butter cultures. Iowa Agri. Exp. Sta. Res. Bull., 191.

KOSER, S. A. 1924 Correlation of citrate utilization by members of the colon-aerogenes group with other differential characteristics and habitat. Jour. Bact., 9, 59–77.

LEVINE, M., EPSTEIN, S. S., AND VAUGHN, R. H. 1934 Differential reactions in the colon group of bacteria. Amer. Jour. Pub. Health, 24, 505–510.

PAINE, F. S. 1927 The destruction of acetyl-methyl-carbinol by members of the colon-aerogenes group. Jour. Bact., 13, 269–274.

SCHMALFUSS, H., AND BARTHMEYER, H. 1929 Diacetyl als aromabestandteil von lebens-und genussmitteln. Biochem. Ztschr., 216, 330–335.

TITTSLER, R. P. 1933 The advantages of O'Meara's modification of the Voges-Proskauer test. Jour. Bact., 25, 40–41.

VAN NIEL, C. B., KLUYVER, A. J., AND DERX, H. G. 1929 Über das butteraroma. Biochem. Ztschr., 210, 234–251.

VISSER'T HOOFT, F., AND DE LEEUW, F. J. G. 1935 The occurrence of acetylmethylcarbinol in bread and its relation to bread flavor. Cereal Chem., 12, 213–229.

WERKMAN, C. H. 1930 An improved technic for the Voges-Proskauer test. Jour. Bact., 20, 121–125.

WILLIAMS, O. B., AND MORROW, M. B. 1928 The bacterial destruction of acetyl-methyl-carbinol. Jour. Bact., 16, 43–48.

THE PRESERVATION OF BACTERIAL CULTURES. I

HARRY E. MORTON AND EDWIN J. PULASKI

Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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The maintenance of a large collection of stock cultures of bacteria for ready availability is a major task. The method of frequent transfer on a suitable culture medium, as commonly employed, requires not only a great expenditure of time, materials and effort, but also involves the possible loss of certain biological, immunological and cultural characteristics; not to mention the occasional loss of the strain through such agencies as contamination, drying of the medium and contact with accumulating metabolites.

It is the purpose of this paper to present (*a*) a review of the literature on the preservation of bacteria, (*b*) a comparison of three of the methods commonly employed for preserving bacterial cultures and (*c*) an emphasis on an inexpensive and practical method for preserving such cultures as are required to be available at all times for frequent subcultures.

To preserve cultures, and at the same time to maintain them in as nearly as possible their original state, various technics have been advocated. All, however, appear to be based upon one of two principles: First, the prevention of slow-drying of the cultures, or, second, the use of rapid desiccation. A review of the literature has revealed that most of the methods of preservation have been tried only on a limited number of bacterial species; practically none have been subjected to critical study. It is difficult to determine, therefore, whether they would be applicable to a large and varied stock culture collection.

It is our object to evaluate three representative methods by testing them against most of the species commonly maintained

by teaching laboratories. One of these, perhaps the first attempt to preserve cultures, consisted of sealing the end of the tube containing the actively-growing organisms. This probably originated with Soyka (1887), Soyka and Kral (1888) and Kral (1889), who grew cultures in specially designed glass cylinders and closed the open ends with glass stoppers. Eisenberg (1888) and Czaplewski (1889) found that ordinary tubes sealed with paraffin worked just as well. Löwi (1918) suggested the use of test tubes fitted with ground glass stoppers which were reinforced with gutta percha paper. Others, employing slight variations of Soyka's method and different culture media, have reported successful preservation of many organisms (Ahuja, 1935; Bolley, 1900; Fiorito, 1925; Kiefer, 1923; Lal, 1920, 1925; Lenskaja, 1931; Martini, 1910; Mereshkowsky, 1909; Morax, 1918; Morton, 1935; Sartory and Maheu, 1909; Schultz, 1901; Shennan and Ritchie, 1907; Totire-Ippoliti, 1923 and Truche and Cotoni, 1912).

To determine the efficacy of Soyka's method, cultures were grown in beef-infusion broth, pH 7.2, sealed off in ampoules and stored at room temperature in the dark. Such ampoules were opened periodically and their contents transferred to fresh media. The sub-cultures were examined for viability, colony form and morphology. The results thus obtained follow.

A. Organisms surviving without change. (*a*) After 45 months: *Bacillus anthracis*, *Bacillus megatherium*, *Bacillus mesentericus*, *Bacillus subtilis*, *Escherichia coli-communior*, *Shigella dysenteriae* (Shiga and Flexner), *Salmonella enteritidis* (Stanley), *Aerobacter aerogenes*, *Diplococcus mucosus* (types A and B), *Eberthella typhosa* (4 strains), *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus albus* (1 strain) and *Staphylococcus aureus* (1 strain). (*b*) After 33 months: *Eberthella typhosa* "R", *Brucella melitensis* "R" and *Micrococcus aurantiacus*.

B. Organisms surviving 30 to 45 months with changes as indicated. *Escherichia coli* "S" ("R" forms), *Escherichia coli* "R" ("S" and small colony forms), *Shigella dysenteriae* (Strong) "S" ("R" forms), *Salmonella enteritidis* "S" ("R" forms), *Salmonella paratyphi* A "S" (extreme "R" and small colony forms),

Salmonella pullorum "R" (mucoid borders), *Eberthella typhosa* "S", 1 strain ("R" forms), *Serratia indica* and *Serratia marcesens*, 4 strains (loss of pigment), *Corynebacterium diphtheriae* "R" (small colonies), *Gaffkya tetragena,* 2 strains (small colonies), *Neisseria catarrhalis* (small colonies), *Proteus vulgaris* (small colonies), *Sarcina lutea* (small colonies and loss of pigment), *Staphylococcus aureus* (aureus and albus type of colonies) and *Vibrio proteus* (small colonies).

C. Organisms not surviving on subculture. (*a*) After 30 to 45 months: *Bacillus novus* (Huss), *Alcaligenes fecalis, Salmonella paratyphi* A, *Brucella abortus* (bovine and porcine), *Chromobacterium violaceum, Corynebacterium hofmannii, Corynebacterium xerose, Micrococcus flavus, roseus* and *tetragenus, Neisseria catarrhalis, Pasteurella pestis* (*caviae*), *Pseudomonas aeruginosa, Sarcina lutea, Staphylococcus albus, Staphylococcus aureus* (1 strain), *Vibrio metchnikovi* and *Vibrio schuylkilliensis.* (*b*) After 15 to 23 months: *Pseudomonas phosphorescens, Alcaligenes bronchisepticus* and *Saccharomyces cerevisiae.*

Summary. Storage of broth cultures in ampoules is not wholly satisfactory. Some species (the species of *Brucella, Pseudomonas phosphorescens,* the species of *Corynebacterium, Pseudomonas aeruginosa* and *Saccharomyces cerevisiae*) did not survive longer than a few months, and many underwent changes in colony form, which persisted. Similar observations on dissociation have been made by earlier workers and more recently by Hadley (1927, 1937). This method, moreover, is not suitable for many species. Of the organisms studied the method was least adaptable to *Corynebacterium diphtheriae.* The "S" form survived only 3 months while the "R" survived 7 months.

With one exception, all the cultures were grown in infusion broth. In the case of one of the diphtheria "R" strains, the organisms were grown in blood infusion broth. In this particular case, the strain was alive at the end of 45 months, whereas in plain infusion broth, the strains were always dead within 9 months.

The observation that the presence of materials such as blood, peritoneal fluid, etc. would greatly prolong the survival time is

not new. Foa (1893) suggested that pneumococci could be preserved by storing septicemic blood. Barnabeo (1896) confirmed Foa's results with pneumococci and streptococci. Puntoni (1923, 1924) employed practically the same technic for successfully preserving the gram-negative intestinal rods. Pergher (1927) and Petragnani (1926) applied the method to an even wider range of bacteria. Fragments of infected tissue (Yourevitch, 1930), diluted bile and infusions of various organs (Totire-Ippoliti, 1924) have, likewise, been suggested.

The second method of preservation of cultures to be described is noteworthy in that it attempts not only to preserve the cultures, but also to keep them readily available at all times for multiple transplantation. The genesis of this method is to be found in the studies of Lumière and Chevrotier (1914). They concluded that gonococci could be maintained viable for several months if the cultures were kept either *in vacuo* or sealed with paraffin oil or vaseline. Ungermann (1918) grew other organisms in dilute, inactivated serum overlayed with sterile paraffin oil. Michael (1921) modified the method by adapting it to ordinary solid media, obtaining good results with a wide range of organisms. Nissle (1925) and Dikomeit (1927) observed that even suspensions of organisms remained viable under a layer of sterile paraffin oil or paraffin-oil lanolin mixtures.

The method has been tested only on a few organisms and, in most instances, for a relatively short period (Birkhaug, 1932; Bruni, 1930; Buschke and Langer, 1921; Daranyi, 1928; Kurobawa, 1927; Olsen, 1920; Parish, 1932; Trozky, 1930 and Truche, 1924). Suitable controls usually have been lacking, and the optimum conditions of storage have not been determined. On the other hand, the method attracts because of its simplicity.

EXPERIMENTAL

To evaluate this method the following experiment was carried out. Organisms were grown on appropriate solid media (infusion agar, "blood" agar, etc.), slanted so as not to give too long a slant. Several cultures were prepared from each strain. After good growth had taken place, one slant was covered with sterile

heavy paraffin oil, or mineral oil, to a height of one centimeter above the top of the slanted surface. For a control, another slant was protected with a rubber cap, a method which had been previously employed with stock cultures. These are illustrated in figure 1. Such pairs of cultures of the same strain were held at the temperature of the incubator, room and refrigerator. To

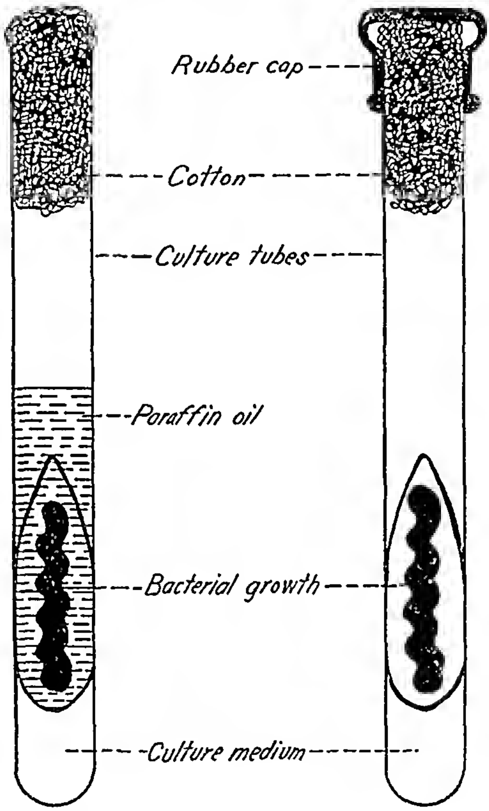

Fig. 1

determine the viability of the cultures under the various conditions, transplants were made from time to time. This was accomplished by fishing off a loopful of the growth, allowing the excess oil to drain off by touching the loop to the inner wall of the tube, then streaking over the surface of fresh medium in the usual manner.

TABLE 1

Preservation of agar slant cultures under paraffin oil

ORGANISM AND STRAIN	REFRIGERATOR TEMPERATURE (8°-11°C.)				ROOM TEMPERATURE				INCUBATOR TEMPERATURE (37°C.)			
	Test		Control		Test		Control		Test		Control	
	Condition	Interval	Condition	Interval	Condition	Interval	Condition	Interval	Condition	Interval	Condition	Interval
		mos.		*mos.*		*mos.*		*mos.*		*mos.*		*mos.*
Aerobacter aerogenes, P 41	+	12*	+	12*	+	21*	Dead	18				
Alcaligenes bronchisepticus					+	30	+	30				
Alcaligenes fecalis, P 61	+	12*	+	12*	+	21	+	21†	+	21	+	18*
Brucella abortus (bovine) P 63	+	12*	Dead	12	+	21*	Dead	18				
Chromobacterium violaceum, P 104	Dead	12	Dead	12	+	18	Dead	12				
					Dead	21						
Corynebacterium diphtheriae, Park 8[1]	+	3	Dead	3	+	36		†	+	12	+	12
	Dead	4							Dead	23		†
Corynebacterium hofmanii, 811[1]					+	30	+	30	+	12*	+	12*
Corynebacterium xerosis, P 83[1]	+	12*	Dead	12	+	33	Dead	33	+	23*	Dead	10
Diplococcus mucosus, P 10	Dead	12	Dead	12	+	21*	Dead	12	Dead	7	Dead	7
Eberthella typhosa, P 12	+	12*	+	12*	+	21	Dead	18				
Escherichia coli-commune "R," P 91	+	9	+	12	+	18	+	18	Dead	9	Dead	9*
	Dead	12	Dead	18	Dead	21	+	21				
Escherichia coli-commune "S," P 3	+	12*	+	12*	+	20*		†	+	9*	+	9*
Hemophilus influenzae[2]									+	1	Dead	1
									Dead	2		
Hemophilus pertussis[2]	+	12	Dead	12	+	12	Dead	12				
					Dead	21						
Monilia albicans[3]									+	7*	Dead	7
Neisseria catarrhalis, P 60	+	21	+	21	+	21	+	21	+	9*	+	9*
Neisseria gonorrheae[1]									+	13	+	3
Neisseria intracelluris[1]											Dead	1
					Dead	3	Dead	3	+	15	Dead	3
Proteus vulgaris, P 11	+	12*	+	18*	+	21	+	21	+	21	+	18
											Dead	21
Pseudomonas aeruginosa	Dead	12	Dead	12	+	21	+	21	+	20*	Dead	9
Pseudomonas phosphorescens					Dead	30	+	30				

Saccharomyces cerevisiae					+	10*	Dead	3	+	4	Dead	3
									Dead	7		
Salmonella enteritidis, P 51	Dead	12	+	12*	+	18	+	18	+	18*	+	12*
Salmonella pullorum "R," P 39	Dead	12	+	12	+	21	+	21	Dead	9	Dead	9
			Dead	18								
Sarcina aurantiaca, P 138	+	12*	+	21	+	18	Dead	18	Dead	9	Dead	9
					Dead	21						
Serratia marcescens, P 135	+	12*	+	12	+	21*	Dead	18				
			Dead	18								
Shigella dysenteriae (Flexner), P 109	+	12*	Dead	12	+	21	Dead	18	+	18*	Dead	18
Shigella dysenteriae (Shiga), P 52	+	12*	+	12	Dead	18	+	18	Dead	21	Dead	21
			Dead	18								
Spirillum rubrum, P 104[1]	Dead	12	Dead	12	+	21	Dead	12				
Staphylococcus albus, P 77	+	21	+	21	+	21	+	21	+	21	+	21
Staphylococcus aureus, P 78	+	12*	+	12*	+	21*	Dead	18	+	21	+	21
Staphylococcus citreus, P 150	+	18	+	18								
	Dead	21	+	21	+	33	+	18*	+	21	Dead	18
Streptococcus cardioarthritidis,[1] P 146	+	9*	Dead	9								
Streptococcus fecalis,[1] P 122	+	20		†	+	19*	Dead	18	+	18	+	18
									+	21*	Dead	21
Streptococcus hemolyticus,[1] P 24	+	18	Dead	18	+	19*	Dead	18	+	12*	Dead	4
Streptococcus indifferens,[1] P 26	+	12*	+	12*	+	12*	+	12*				
Streptococcus pneumoniae "M," type I[1]	+	12*	+	12*	+	12*		†				
Streptococcus pneumoniae "S," from type I[1]	+	9*	Dead	7								
Streptococcus pneumoniae "M," type II[1]	+	20*	Dead	9	+	12*	Dead	12				
Streptococcus pneumoniae "S" from type II[1]	+	12*	Dead	12	+	12*	Dead	12				
Streptococcus pneumoniae "M" type III[1]	+	20*	Dead	9	+	12*		†				
Streptococcus viridans,[1] P 25	+	12*	+	12*	+	12*	Dead	12				
	+	20*		†								
Vibrio metchnikovi, P 45	+	18	+	18	+	18	+	18	+	7*	Dead	7
	Dead	21	+	21*	Dead	21	Dead	21				
Vibrio schuylkilliensis, P 8	+	18	+	18	+	18	+	18	+	9*	Dead	9
	Dead	21	+	21	Dead	21	+	21				

+ indicates culture alive.
* Slant washed for making test.
† Culture overgrown with a mold.
[1] Culture maintained on blood agar.
[2] Culture maintained on chocolate agar.
[3] Culture maintained on glucose agar.

Attention is directed to the following points: (*a*) Unless the oil is well above the uppermost level of the medium, the medium tends to dry out, separate from the wall of the tube and float to the surface of the oil, in which event the organisms are usually found dead. (*b*) The quality of the oil[1] is very important, as any rancidity or toxic substance is harmful to the organisms. (*c*) It is preferable to sterilize the oil in the hot air oven at 150° to 170°C. for one hour; for during autoclaving moisture becomes mixed with the oil, giving it a milky appearance. (*d*) Some precaution is necessary in the flaming of the loop after it has been withdrawn from the oil, since plunging directly into the flame results in spattering. This may be prevented by warming the loop very gently before heating to redness, or by plunging into a beaker of boiling water, then flaming to redness in the usual manner. The technics customarily employed with acid-fast organisms are satisfactory.

The significant results thus far obtained are presented in table 1.

Summary. Forty-four strains, representing a wide variety of bacterial species, have been tested:

Test culture alive, control culture dead	29
Test culture alive, control culture alive	10
Test culture dead, control culture alive	5
Totals: Test cultures alive	39
Control cultures alive	15

The five instances where the control culture survived longer than the culture under oil were *Escherichia coli* "R", *Shigella dysenteriae* (Shiga), *Pseudomonas phosphorescens*, *Vibrio metchnikovi* and *Vibrio schuylkilliensis*. The viable cultures are being held for further observations. The rubber cap on the control cultures probably accounts for the long survival period of the controls, for Birkhaug and Parish report all their controls dead within four weeks. Also we were unable to confirm the favor-

[1] Mineral oil (Heavy), Parke, Davis and Company has proved very satisfactory.

able results of these authors in regards to *Hemophilus influenzae*. No single temperature is optimum for storage of all bacteria. Room temperature appears to be optimum in the majority of cases, the exceptions being certain of the *Neisseria*, *Hemophilus* and *Streptococcus* groups.

The first two methods described had as their basis the prevention of slow evaporation of the cultures. In contrast, the third method employs the principle of rapid desiccation.

Slow desiccation in air appears to have a decidedly lethal effect. For example, organisms left to dry on glass slides survive no longer than a few days (*Brucella abortus* (Cameron, 1932); *Bacillus anthracis*, *Escherichia coli*, *Corynebacterium diphtheriae*, *Eberthella typhosa*, *Saccharomyces cerevisiae* and *Staphylococcus aureus* (Thurn, 1914); *Vibrio cholerae* (Thurn, 1914; Kitasato, 1889); *Neisseria intracellularis* (Bettencourt and Franca, 1904)). The presence of extraneous material such as silk (Kitasato, 1889); sputum (Bordoni-Uffreduzzi, 1891) and various nutritive substances (Foa and Bordoni-Uffreduzzi, 1887; Abel, 1893, 1897; Latapie, 1918; Winslow and Brooke, 1927; Cameron, 1932) greatly alters the survival period.

Kitasato in 1889, working with *Vibrio cholerae*, observed that the organisms survived longer when desiccator-dried than when air-dried. Germano (1897 a and b) demonstrated quantitatively that diphtheria bacilli, streptococci and pneumococci survived longer when dried in a desiccator over H_2SO_4 than when room-dried. Ficker (1898) was the first to make a critical study of other factors necessary for the preservation of bacteria by desiccation. He concluded that most important were (*a*) the mass and nature of the dried cultures, and (*b*) the menstruum in which the organisms were suspended. In 1908 he reported on the nature of the suspending medium, finding that milk, serum, bouillon, saliva, distilled water, physiological salt solution and urine protected the organisms in the order listed; milk and inactivated serum being best. He also pointed out that another factor in the survival of bacteria is the change in osmotic conditions brought about in drying. Kirstein (1900) found alternate humidity and dryness more destructive than constant dryness.

He also observed that the dried bacteria lived longer at refrigerator temperature than at room temperature.

Heim (1905, 1907) was the first to report the successful use of drying in a desiccator over a dehydrating agent as a method for preserving a variety of cultures, some surviving two years. He later (1922) used small sterile test tubes instead of glass slides. Brown (1925) reported preserving pneumococci and streptococci by drying on cover-slips in a desiccator over $CaCl_2$. The technic was soon modified, strips of sterile filter paper being substituted for the glass cover-slips and pint milk bottles being used instead of a desiccator. In 1926 he further refined the technic by employing, in some cases, small sterile test tubes instead of the pint milk bottles. In a later report (1932) some strains were reported as remaining viable for as long as 12 years. Harris and Lange (1933), using the method of Brown, found that acid-fast organisms (31 different strains) could be preserved for at least 11 months. Leifson (1936), using a slight modification of Brown's technic, did not obtain commendable results.

Patella (1888) observed that pneumococci dried at 38°C. did not maintain their virulence or viability as long as when dried at a lower temperature (17°C.), but it was not until 1909 that freezing was recommended as a preliminary step to desiccation (Shackell). Hammer (1911) modified Shackell's method in that he dipped strips of paper in bouillon cultures before the freezing and drying process.

Rogers (1914) applied the principle of freezing and drying of bacterial cultures on a large scale. The cultures were frozen by a salt-ice mixture or by means of carbon-dioxide snow. Good results were obtained with the lactic-acid group and the colon group of organisms, questionable results being obtained with yeasts. Rogers found that the loss of viability of the dried cultures was very slow at low temperatures (0°C. or lower), but became more rapid as the temperature of storage increased. This is a confirmation of Ficker's (1898) observations. More cells remained viable in cultures stored *in vacuo* than in hydrogen, carbon dioxide, nitrogen, oxygen or air. The various gaseous environments were least detrimental in the order named. He

suggested that the method could be applied for the preservation of stock cultures.

Swift (1921) applied the method of drying cultures from the frozen state to streptococci, pneumococci, meningococci and influenza, typhoid, paratyphoid and dysentery bacilli with good results. Otten (1927, 1930) showed that freezing could be eliminated from Swift's technic and he was able thereby to preserve a wide variety of pathogenic bacteria. Pauli (1932) also successfully preserved many cultures without freezing them prior to desiccation, recommending suspension of the organisms in sterile normal horse serum. In 1935 two methods (Elser, Thomas and Steffen, and Flosdorf and Mudd, 1935, 1936) were reported for the preservation of biological products, including microorganisms, by drying from the frozen state. That of Flosdorf and Mudd employed dry-ice for the initial freezing. Elser, Thomas and Steffen reported meningococci and gonococci alive after storage *in vacuo* for 18 years. Rake in 1935 reported the successful preservation of meningococci for periods ranging from 3 to 5 months by freezing the organisms in a dry-ice freezing mixture and drying in a vacuum. In 1937, Swift modified his method so as to employ dry-ice for the freezing agent. Roe (1936) preserved 16 varieties of anaerobes by drying them on strips of filter paper from the frozen state and maintaining in sealed test tubes with a freshly heated piece of $CaCl_2$, thus combining the technics of Brown and Swift.

Experimental

This work was begun in 1932 and of the methods reported in the literature, which employ rapid desiccation, we chose that of Swift (1921). The technic employed was as follows: Heavy suspensions of young bacterial cells were dispensed in amounts of 0.25 cc. into sterile agglutination tubes. These were placed in a salt-ice mixture until frozen (those cultures designated in table 2 by an asterisk were frozen in a dry-ice acetone mixture), then transferred to a desiccator containing a layer of glycerol kept cold by being surrounded with a salt-ice mixture. A dish containing P_2O_5 was placed above the tubes in the desiccator,

TABLE 2

Preservation of bacteria by the rapid drying technic of Swift

ORGANISM AND STRAIN	INTERVAL AFTER DRYING	CONDITION
	months	
Actinomyces casei, P 133*	3	+
Aerobacter aerogenes, P 41	58	+
Alcaligenes bronchiseptica	40	+
Alcaligenes fecalis, P 61	37	+
Bacillus anthracis, "R," P 60	28	+
Bacillus megatherium, P 97*	24	+
Bacillus mesentericus, P 112*	24	+
Bacillus novus (Huss), P 102*	45	+
Bacillus subtilis, P 7*	17	+
Brucella abortus (bovine) P 63	20	+
Brucella abortus (caprine) P 80	24	+
Brucella abortus (porcine) P 65	22	+
Corynebacterium diphtheriae, Park 8, O 4	24	+
Corynebacterium xerosis, P 83	36	+
Diplococcus mucosus, types A and B	37	+
	58	Dead
Eberthella typhosa, P 16*	45	+
Eberthella typhosa, P 12, 17, 20, 32	58	+
Escherichia coli-commune, "S," P 3	58	+
Escherichia coli-communior, P 101*	4	+
Hemophilus influenzae, P 67	27	+
Lactobacillus acidophilus "S," P 117*	15	+
Micrococcus aurantiacus, P 103*	4	+
Micrococcus roseus, P 100*	16	+
Micrococcus tetragenus, P 40	36	+
Neisseria catarrhalis, P 66*	4	+
Pasteurella pestis (caviae), P 71	58	+
Proteus mirabilis, P 98*	16	+
Proteus vulgaris, P 44*	25	+
Pseudomonas aeruginosa, P 2	58	+
Pseudomonas phosphorescens	12	+
Salmonella enteritidis, P 51	56	+
Salmonella paratyphi A, P 54, 22, 23, 55, 56	36	+
Salmonella paratyphi A, P 56	54	Dead
Salmonella paratyphi A, P 57	57	+
Salmonella paratyphi A, P 58	16	+
	37	Dead
Salmonella paratyphi B, P 33, 34, 35, 36	36	+
Salmonella paratyphi B, P 59	57	+
Salmonella paratyphi B, P 113*, 114*	45	+

TABLE 2—*Concluded*

ORGANISM AND STRAIN	INTERVAL AFTER DRYING	CONDITION
	months	
Salmonella pullorum, "R," P 39	58	+
Salmonella pullorum, "R," P 47	4	+
	37	Dead
Serratia marcescens, P 4*	45	+
Serratia marcescens, P 4	44	+
Shigella dysenteriae (Flexner) P 21	54	+
Shigella dysenteriae (Flexner) P 109*	57	Dead
Shigella dysenteriae (Shiga) P 52	36	+
	57	Dead
Shigella dysenteriae (Strong) P 53	57	+
Shigella dysenteriae (Y) P 107*	4	+
Staphylococcus albus, P 77	36	+
Staphylococcus aureus, P 5, 37	36	+
Staphylococcus aureus, P 37	58	Dead
Staphylococcus aureus, P 38	58	+
Staphylococcus aureus, P 78	36	+
	58	Dead
Streptococcus fecalis, P 122*	25	+
Streptococcus hemolyticus, P 24	43	+
	57	Dead
Streptococcus hemolyticus, P 73, 74	58	+
Streptococcus indifferens, P 26	36	+
Streptococcus pneumoniae, type I, P 21	58	+
Streptococcus pneumoniae, S from type I	37	+
Streptococcus pneumoniae, type II, P 28	54	+
Streptococcus pneumoniae, S from type II	37	+
	57	Dead
Streptococcus pneumoniae, type III, P 29	36	+
	57	Dead
Streptococcus pneumoniae, S from type III	17	+
	57	Dead
Streptococcus pneumoniae, type V, P 30	60	Dead
Streptococcus pneumoniae, type VI, P 31	60	+
Streptococcus viridans, P 25	57	+
Vibrio metchnikovi, P 45	4	+
	37	Dead
Vibrio schuylkilliensis, P 8	4	Dead

* Cultures frozen in dry ice-acetone mixture (May, 1933).
+ indicates cultures alive and typical.

the lid placed in position and connected to a vacuum pump (Cenco Hyvac). After the desiccator had been evacuated for 1 to 2 hours, the pump was disconnected and the desiccator placed in the refrigerator. The next day the dried cultures were removed and the open ends of the tubes sealed with paraffin. The preserved cultures were kept at room temperature and in the dark. Those tubes showing a gummy residue were discarded. The results thus far obtained are given in table 2.

Summary. The method has been found to be reasonably trustworthy. However, a number of the strains, which were viable when tested after 3 years, were found dead when duplicate tubes were tested after a period of 57 months (*Shigilla dysenteriae*, *Diplococcus mucosus*, *Salmonella paratyphi* A, *Staphylococcus aureus*, *Streptococcus hemolyticus*, *Streptococcus pneumoniae*, *Vibrio metchnikovi*). Due to the many factors involved in this method, it is impossible to state the reason for the failure of survival of some of the strains. At first it was believed that the breakage of the paraffin seal due to changes in room temperature, especially during the summer months, was responsible for the death of certain of the cultures. However, in other experiments where one portion of the tubes was hermetically sealed, and the other sealed with paraffin in the usual manner, no perceptible difference was noticed in the survival period.

DISCUSSION

From the preliminary studies herein reported, many interesting points have arisen. The preservation of bacterial cultures in ampoules is of little value, if the original characteristics of the strain are to be preserved. Some organisms live only a short time and frequently the cultures undergo dissociation. The method is costly, as culture tubes are lost when they are sealed. It is somewhat dangerous because of the possible shattering of the ampoule when opened. Once an ampoule is opened, a fresh culture has to be sealed off or the contents of the opened ampoule transferred to another tube and resealed.

Preservation of cultures under paraffin oil has many distinct advantages. The cultures are available at all times; numerous

subcultures can be made without the necessity of discarding the original culture. The method eliminates the use of rubber caps on the culture tubes, thereby bringing about an economy. It, likewise, eliminates the use of wax, cements, etc. which are difficult to remove. It reduces the frequency of contamination to practically nil, especially with molds. No preliminary treatment, such as growth in large amounts, centrifugation, dispensation into special tubes or onto filter paper, is necessary. No special apparatus, such as desiccators and vacuum pumps, is needed. Single colonies or single colony variants are easily preserved without disturbing the stage of development. Practically all the organisms tested live longer under paraffin oil than in the unprotected control cultures. Although good results were not obtained with the one strain of *Shigella dysenteriae* (Shiga) cited in table 1, other strains of the dysentery bacillus and other strains of the Shiga bacillus are being maintained under oil without difficulty. Even in the case of the influenza bacillus where the period of survival under oil was only two months, this was twice as long as in the control culture. With the exception of the influenza bacillus, it is only necessary to make transfers every six months or even once a year. Bucher (1937) states that he maintained a large collection of freshly isolated strains of meningococci and gonococci by making transfers every six months and preserving under paraffin oil.

Preservation of cultures in the dried state by Swift's method has the advantage that many organisms apparently survive longer in the dried state than by other methods of preservation. The cultures can be stored in small containers, which is an aid if storage space is at a minimum or if it is necessary to transport the cultures, and the dry state of the cultures makes them more adaptable to transportation than if they were in the fluid state. The disadvantages of the method are many. Once a tube of the dried culture is opened for subculturing, the culture is lost as far as preservation is concerned, unless one has many duplicate cultures or goes to the trouble of freezing and drying additional cultures from time to time. The method requires growth of the organisms in large quantities. In some cases, such as

growth of unstable variants, this is not always practical. The method requires a great deal of manipulation, such as centrifugation, dispensation into tubes, freezing and sealing of the tubes. Special apparatus is also required. When removed from the desiccator, it is necessary to wipe the glycerol from the exteriors of the tubes. This is messy. Sealing of the tubes is not without its difficulties. Paraffin and other of the various preparations for that purpose contract and crack due to the cool temperature of the refrigerator and this results in the loss of the cultures. Likewise, the high temperatures encountered in the summer time cause a damage to the seals with subsequent loss of the cultures. It is not practical by the present method to preserve large numbers of cultures individually in the dried state under a vacuum. In view of the fact that numerous workers have reported cultures alive in ampoules for many years, Swift's method has not been under investigation long enough to warrant a definite statement on the longevity of bacteria thus preserved. One is appalled at the lack of critical data on the preservation of cultures by this method. Methods which have been in use for just a few years enjoy such claims as "the cultures would probably keep indefinitely if the seals remained intact." Only by quantitative studies, which have been lacking, will it be possible to venture a definite statement.

SUMMARY

In laboratories where it is necessary to make frequent transplants from stock cultures, a very practical method is to maintain them under sterile paraffin oil. The points in favor of the method are many. (*a*) It greatly reduces the frequency of contamination, especially with molds, thus permitting cultures to be maintained with greater success in surroundings which are not conducive to precise bacteriological work. (*b*) No preliminary treatment of the cultures is necessary. (*c*) Practically all the organisms tested live longer under oil than in the control tubes. (*d*) Changes in cultural and biochemical characteristics—other than the sometimes prolonged lag phase of growth on subculturing—have not been observed. (*e*) The cultures are

available at all times for transplantation without interfering with the preservation of the stock culture. (*f*) The method is applicable to single colonies or mass cultures. (*g*) It is especially advantageous in working with unstable variants, where occasional transferring to fresh media or growth in mass culture results in a change in the developmental stage of the strain. (*h*) No seals, such as rubber caps, waxes, cements, etc. are needed for the culture tubes. (*i*) No special apparatus is required, such as a centrifuge, desiccator or vacuum pump.

The method of preserving cultures by sealing the tubes in a flame is of questionable practical value, if the original characteristics of a culture are to be preserved. Cultures which remain viable in ampoules frequently show only a few viable organisms when transferred to fresh media and often the viable organisms have dissociated. Other disadvantages are that the cultures are not easily available for subculturing and there is destruction of culture tubes.

Preservation of cultures in the dried state has the advantages that (*a*) less space is required for storage, (*b*) the cultures are more easily transported and (*c*) certain immunological properties apparently are maintained. The disadvantages are that the cost of the special equipment (centrifuge, vacuum pump, etc.) is prohibitive in some laboratories; likewise, the time and effort which must be expended if a large collection of cultures is to be so preserved. Many of the technics for preserving cultures in the dried state are impractical and some technics are more destructive to certain micro-organisms than if the organisms were kept on the original culture medium.

Lack of critical systematic studies upon, and the possibilities of, the method of preservation in the dried state are the basis of additional studies which are now in progress.

REFERENCES

ABEL, R. 1893 Beitrag zur Frage von der Lebensdauer die Diphtheriebacillen. Centralbl. f. Bakt., O., 14, 756-61.

ABEL, R. 1897 Zur Kenntnis des Pestbacillus. Centralbl. f. Bakt., O., 21, 497-517.

AHUJA, M. L. 1935 A short note on viability of bacterial cultures. Indian Jour. Med. Res., 22, 477-8.

BARNABEO, G. 1896 Sulla conservazione della vitalita e virulenza dello pneumococco di Fränkel e dello streptococco del Fehleisen. La Riforma Medica, 12, 242-4.

BETTENCOURT, A., UND FRANCA, C. 1904 Ueber die Meningitis cerebrospinalis epidemica und ihren specifischen Erreger. Ztschr. f. Hyg. u Infektionskr., 46, 463-516.

BIRKHAUG, K. E. 1932 Preservation of bacterial cultures under liquid paraffin Science (N.S.), 76, 236-7.

BOLLEY, H. L. 1900 The duration of bacterial existence and trial environments Centralbl. f. Bakt., II. 6, 33-8.

BORDONI-UFFREDUZZI, G. 1891 Sulla resistenza del virus pneumonico Negli sputi. Arch. per le Sc. Med., 15, 341-8.

BROWN, J. H. 1925 The preservation of bacteria in vacuo, I. Abstracts Bact., 9, 8.

BROWN, J. H. 1926 Vacuum tubes for the storage and shipment of bacteria Science (N.S.), 64, 429-30.

BROWN, J. H. 1932 The preservation of bacteria in vacuo, II. Jour. Bact., 23, 44.

BRUNI, E. 1930 La conservazione in colture del meningococco. Ann. di Med Nav. e Colon., 36, 396-8.

BUCHER, C. J. 1937 Personal communication.

BUSCHKE, A., UND LANGER, E. 1921 Ueber die Lebensdauer und anaërobe Züchtung der Gonokokken. Deutsche Med. Wchnschr., 47, 65-7.

CAMERON, H. S. 1932 The viability of Brucella abortus. Cornell Vet., 22, 212-24.

CZAPLEWSKI, E. 1889 Zur Anlage bakteriologischer Museen. Centralbl. f. Bakt., O., I, 6, 409-11.

DARÁNYI, J. VON 1928 Beitrag zur Konservierung von Bakterienkulturen mit Paraffin. Centralbl. f. Bakt., O., 108, 160-2.

DIKOMEIT, B. 1927 Ueber ein einfaches Verfahren zur Konservierung lebender Bakterienkulturen. Centralbl. f. Bakt., O., I, 101, 290-301.

EISENBERG, J. 1888 Bemerkungen über Kartoffeldauerkulturen nach der Methode des Prof. J. Soyka. Centralbl. f. Bakt., O., I, 3, 216-7.

ELSER, W. J., THOMAS, R. A., AND STEFFEN, G. I. 1935 The desiccation of sera and other biological products (including microorganisms) in the frozen state with the preservation of the original qualities of products so treated. Jour. Immunol., 28, 433-73.

FICKER, M. 1898 Über Lebensdauer und Absterben von pathogenen Keimen Ztschr. f. Hyg. u. Infektionskr., 29, 1-74.

FICKER, M. 1908 Über die Resistenz von Bakterien gegenüber dem Trocknen Ztschr. f. Hyg. u. Infektionskr., 59, 367-78.

FIORITO, G. 1925 Sulla vitalità di alcune culture di germi asporigeni. Ann d'ig., 35, 702-6.

FLOSDORF, E. W., AND MUDD, S. 1935 Procedure and apparatus for preservation in "Lyophile" form of serum and other biological substances Jour Immunol., 29, 389-425.

FLOSDORF, E. W., AND MUDD, S. 1936 Rapid drying of serum and microorganisms from the frozen state for preservation. Rep. Proc. 2nd Internat Congress Microbiol. (London), p. 45.

Foa, P. 1893 Sulla infezione da diplococco lanceolato. Arch. per le Sc. Med., 17, 381–421.

Foa, P., e Bordoni-uffreduzzi, G. 1887 Sulla eziologia della meningite cerebrospinale epidemica. Arch. per le Sc. Med., 11, 385–421.

Germano, E. 1897a Die Uebertragung von Infektionskrankheiten durch die Luft. II, Mittheilung: Die Uebertragung der Diphtherie durch die Luft. Ztschr. f. Hyg. u. Infektionskr., 25, 439–52.

Germano, E. 1897b Die Uebertragung von Infektionskrankheiten durch die Luft. III, Mittheilung: Die Uebertragung des Erysipels, der Pneumonie und anderer Streptokokkeninfectionen durch die Luft. Ztschr. f. Hyg. u. Infektionskr., 26, 66–89.

Hadley, P. 1927 Microbic dissociation. Jour. Infect. Dis., 40, 1–312.

Hadley, P. 1937 Further advances in the study of microbic dissociation. Jour. Infect. Dis., 60, 129–92.

Hammer, B. W. 1911 A note on the vacuum desiccation of bacteria. Jour. Med. Res., 24, 527–30.

Harris, M. M., and Lange, L. B. 1933 A note on the preservation of acid-fast bacteria in vacuo. Jour. Lab. and Clin. Med., 18, 1066–7.

Heim, L. 1905 Die Widerstandsfähigkeit verschiedener Bakterienarten gegen Trocknung und die Aufbewahrung bakterienhaltigen Materials insbesondere beim Seuchendienst und für geriehtlichmediciniscbe Zwecke. Ztschr. f. Hyg. u. Infektionskr., 50, 123–38.

Heim, L. 1907 Ueber Pneumoniecoccen. Deutsche Med. Wchnschr., 33, 1587–8.

Heim, L. 1922 Lehrbuch der Bakteriologie, Aufl. 7, 228, (Enke, Stuttgart).

Kiefer, K. H. 1923 Ein Beitrag zur Lebensfähigkeit der Bakterien. Centralbl. f. Bakt., O., 90, 1–5.

Kirstein, F. 1900 Ueber die Dauer der Lebensfähigkeit der mit feinsten Tröpfschen verspritzten mikroorganismen. Ztschr. f. Hyg. u. Infektionskr., 35, 123–64.

Kitasato, S. 1889 Die Widerstandfähigkeit der Cholerabacterien gegen das Eintrocknen und gegen Hitze. Ztschr. f. Hyg. u. Infektionskr., 5, 134–40.

Kral, F. 1889 Weitere Vorschläge und Anleitungen zur Anlegung von bacteriologischen Museen. Ztschr. f. Hyg. u. Infektionskr., 5, 497–505.

Kurosawa, A. 1927 Ein Beitrag zur Konservierung lebender Bakterien. Tohoku Jour. Exper. Med., 9, 70–2.

Lal, N. 1920 The viability of bacterial cultures. Indian Jour. Med. Res., 8, 728–30.

Lal, N. 1925 The viability of bacterial cultures (2) Indian Jour. Med. Res., 13, 189–90.

Latapie, A. 1918 Contribution à l'étude du bacilli de Pfeiffer. Compt. rend. soc. biol., 81, 833–5.

Leifson, E. 1936 The preservation of bacteria by drying in vacuo. Amer. Jour. Hyg., 23, 231–6.

Lenskaja, G. von, et al. 1931 Lebenserhaltung des B. pestis bei vieljariger Aufbewahrung auf Nahrboden. Vestnik Mikr. Epidemiol. i, Parazitol., 10, 156–8.

Löwi, E. 1918 Verschlusshülsen für Kulturröhrchen und Vorratsgefässe zur Verhinderung der Verdunstung. Centralbl. f. Bakt., O., I, 81, 493–5.

Lumière, A., and Chevrotier, J. 1914 Sur la vitalité des cultures de gonocoques. Compt. rend. acad. sci., 158, 1820–1.

Martini 1910 Über hohe Grade von Lebensdauer bei Typhus—, Paratyphus B, Aertryck, Gärtnerschen, Enteritis—, und bei Ruhr-Bakterien des Typhus Shiga-Kruse, Flexner und Y. Ztschr. f. Hyg. u. Infektionskr., 65, 121–6.

Mereshkowsky, S. S. 1909 Untersuchung einer achtjärigen Bouillon Kultur des Ziesel typhus bacillus (B. typhi spermophilorum). Centralbl. f. Bakt., O., I, 51, 1–6.

Michael, M. 1921 Die Konservierung schwer haltbarer Bakterienkulturen, insbesondere des Gonococcus (Modifikation der Ungermannschen Methode). Centralbl. f. Bakt., O., I, 86, 507–10.

Morax, V. 1918 A propos de la vitalité du gonocoque. Ann. Inst. Pasteur, 32, 471–5.

Morton, H. E. 1935 *Observations on the life history of the diphtheria bacillus* Thesis, University of Michigan, Ann Arbor, Michigan.

Nissle, A. 1925 Weiteres über Grundlagen und Praxis der Mutaflorbehandlung. Deutsche Med. Wchnschr., 51, 1809–13.

Olsen, O. 1920 Untersuchungen über den Pfeifferschen Influenzabacillus während der Grippepandemie, 1918–19–20. II. Centralbl. f. Bact., O., 85, 12–27.

Otten, L. 1927 Trockenlymphe. Ztschr. f. Hyg. u. Infektionskr., 107, 677–96.

Otten, L. 1930 *Die Trockenkonservierung von pathogenen Bakterien.* Centralbl. f. Bakt., O., 116, 199–210.

Parish, H. J. 1932 Preservation of cultures under liquid paraffin. Jour. Path. and Bact., 25, 143–4.

Patella, V. 1888 Sulla pneumonite cruposa. Atti della Reale acad. Med. di Roma, 4, series 2, 447–74.

Pauli, P. 1932 A propos d'un procidi facile pour la conservation des microorganismes à l'état de vie latente. Soc. internaz di microbiol. Boll. d. sez. ital., 4, 239–43.

Pergher, G. 1927 Le conservazione dei microbi asporigeni in sangue. Ann. d'ig., 37, 438–45.

Petragnani, G. 1926 La lunga conservazione degli stipiti batterici nel sangue di cavia. Gior. di Batteriol. e. Immunol., 1, 364–7.

Puntoni, V. 1923 Sopravvivenza decennale di alcuni microbi del gruppo tifocoli conservati in liquidi organici. Ann. d'ig., 33, 165–8.

Puntoni, V. 1923 II. Sopravvivenza decennale del vibrione colerico conservato in liquido peritoneale. Ann. d'ig., 33, 321–4.

Puntoni, V. 1924 La sopravvivenza dei microbi asporigeni. Policlinico, sez. prat., 31, 219–21.

Rake, G. 1935 Viability and virulence of frozen and dried cultures of meningococcus. Proc. Soc. Exper. Biol. and Med., 32, 975–7.

Roe, A. F. 1936 Preserving anerobes by desiccation. Jour. Bact., 31, 25–9.

Rogers, L. A. 1914 The preparation of dried cultures. Jour. Infect. Dis., 14, 100–23.

SARTORY, A., ET MAHEU, J. 1909 Durée de survie chez quelques Bactéries. Compt. rend. soc. biol., 66, 968–70.

SCHULTZ, N. K. 1901 Über die Lebensdauer von Bacillus pestis hominis in Reinkulturen. Centralbl. f. Bakt., 29, 169–74.

SHACKELL, L. F. 1909 An improved method of desiccation with some applications to biological problems. Amer. Jour. Physiol., 24, 325–40.

SHENNAN, T., AND RITCHIE, W. T. 1907 A bacteriological investigation of epidemic cerebrospinal meningitis. Jour. Path. and Bact., 12, 456–86.

SOYKA, J. 1887 Ueber ein Verfahren, Dauerpräparate von Reinculturen auf festem Nährboden herzustellen. Centralbl. f. Bakt., O., I, 1, 542–4.

SOYKA, J., u. KRAL, F. 1888 Vorschläge und Anleitungen zur Anlegung von bacteriologeschen Museen. Ztschr. f. Hyg. u. Infektionskr., 4, 143–50.

ST. JOHN-BROOKS, R., AND RHODES, M. 1936 Some useful media for the preservation of stock cultures. Notes on high vacuum desiccation. Rep. Proc. 2nd Internat. Congress Microbiol. (London), p. 43.

SWIFT, H. F. 1921 The preservation of stock cultures of bacteria by freezing and drying. Jour. Exper. Med., 33, 69–75.

SWIFT, H. F. 1937 A simple method for preserving bacterial cultures by freezing and drying. Jour. Bact., 33, 411–21.

THURN, O. 1914 Ueber die Lebensfähigkeit an Objektträgern angetrockneter ungefärbter und gefärbter Bakterian. Centralbl. f. Bakt., 74, 81–90.

TOTIRE-IPPOLITI, P. 1923 Ulteriori osservazioni sulla vitalita dei microorganismi nelle colture in tubi chiusi alla fiamma. La Nuova Veterinaria, 159–62.

TOTIRE-IPPOLITI, P. 1924 Osservazioni su germi del gruppo tifo-coli conservati in infusi organici. Policlinico. sez. prat., 31, 1227–9.

TROZKY, W. 1930 Uber die Konservierungs methode der Mikrobenkulturen unter Vaselinol. (Laboratoranja Praktika, 1929, Nr. 6.) Centralbl. f. Bakt., R., 99, 189.

TRUCHE, C. 1924 Moyen simple et pratique de conservation des germes. Ann. Inst. Pasteur., 38, 516–9.

TRUCHE, C., ET COTONI, L. 1912 Etudes sur le pneumocoque. II. Conservation de la virulence des pneumocoques humains pour la souris. Ann. Inst. Pasteur, 26, 1–5.

UNGERMANN, E. 1918 Eine einfache Methode zur Gewinnung von Dauerkulturen empfindlicher Bakterienarten und zur Erhaltung der Virulenz tierpathogener Keime. Arb. a. d. Reichsgesundheitsamte, 51, 180–99.

WINSLOW, C.-E. A., AND BROOKE, O. R. 1927 The viability of various species of bacteria in aqueous suspensions. Jour. Bact., 13, 235–43.

YOUREVITCH, V. 1930 Culture latente pour la conservation des pneumocoques et d'autres microbes au point de vue de leur vitalité et de leur virulence. Compt. rend. soc. biol., 103, 320–1.

HYDROGEN SULPHIDE STUDIES

I. Detection of Hydrogen Sulphide in Cultures

CHARLES A. HUNTER and H. GILBERT CRECELIUS

Department of Bacteriology and State Health Laboratory, University of South Dakota

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A considerable number of investigators have proposed methods for the detection of hydrogen sulphide in cultures. Media made from different brands of peptone as well as the addition of sulphur compounds, such as cystine, sodium sulphite, sodium sulphate, taurin, thiourea, etc., have been tried. Burnet and Wissenbach (1915), Kligler (1917), Jordan and Victorson (1917), Thompson (1921), Tilley (1923), Mulsow and Paine (1925), Bailey and Lacey (1927), and others added a lead salt to the medium to serve as an indicator for the presence of hydrogen sulphide. Filter papers impregnated with lead acetate or lead carbonate have been used by Tanner (1917), Meyer (1920), Wilmet (1927), Zobell and Feltham (1934) and others. The use of iron salts for the detection of hydrogen sulphide has been recommended by Wilson ($FeCl_3$) (1923), Beckwith and Moser ($FeCl_3$) (1932), Schunk ($FeSO_4$) (1924) and Levine and coworkers (ferric citrate) (1932). The addition of bismuth carbonate to the culture medium was recommended by Pacheco and Mello (1932). In 1934 Zobell and Feltham compared lead, iron and bismuth as indicators for detecting hydrogen sulphide formation and concluded that iron was more sensitive and less toxic than either lead or bismuth. They recommend, however, the use of lead acetate papers when testing new and unknown pure cultures of bacteria.

In considering the detection of hydrogen sulphide produced by cultures two main problems present themselves. First, the

selection of a sensitive indicator of low toxicity and distinct color change; second, the selection of a medium suitable for the growth of the organisms, and containing an available source of sulphur. An indicator may be of very low toxicity but when added to a medium it may combine with some of the ingredients producing a precipitate, thereby increasing toxicity and decreasing sensitivity.

All of the heavy metals will react with hydrogen sulphide but only a few are desirable for bacteriological work. Those commonly used as indicators are lead and iron salts. Theoretically, iron is more sensitive to hydrogen sulphide in an alkaline menstruum than in an acid one. As most of the culture media are slightly acid or neutral, it follows that the sensitivity of iron is decreased and minute quantities of hydrogen sulphide will not be detected. Therefore, an indicator which will be sensitive in the acid range is desirable.

Theoretically, bismuth is a metal which is sensitive in an acid menstruum. The difficulty in the use of bismuth is that most bismuth compounds are insoluble and therefore not applicable to bacteriological work. There are, however, several soluble bismuth compounds which are of low toxicity and which are good indicators for the detection of hydrogen sulphide.

PREPARATION OF BISMUTH INDICATOR

A soluble bismuth compound, suitable for bacteriological work and commonly known as "bismuth liquor," is prepared as follows:

Place 3 grams bismuth citrate, Merck U. S. P. VIII, in a glass stoppered bottle and add about 10 cc. of distilled water. Mix well and add approximately 1 cc. of NH_4OH sp. gr. 0.90. The bismuth citrate dissolves quickly forming a clear, colorless solution. If it does not clear up readily, gentle heat will increase the solubility. Add distilled water to bring the volume up to 100 cc., one-half (0.5) cc. of the bismuth liquor is added to each 100 cc. of medium.

The bismuth liquor prepared according to the above directions will remain perfectly clear for several weeks and in that condition can be used with satisfactory results. Should the solution become turbid or opalescent the solution should be discarded and a fresh solution prepared.

In place of the bismuth liquor 16 mgm. of Bismuth and Ammonium Citrate, Merck U. S. P. IX, may be added to each 100 cc. of medium.

COMPARISON OF THE SENSITIVITY OF IRON AND BISMUTH SALTS TO H_2S

Several experiments were performed to determine the relative sensitivity of ferric ammonium citrate and bismuth liquor. A series of test tubes were prepared containing 5 cc. of various buffers ranging from pH 6.0 to 9.0 in steps of 0.5 pH. To each tube of the buffer series was added 2.5 mgm. of ferric ammonium citrate and to each tube of a second series 0.125 cc. of bismuth liquor. The tubes in each series were then titrated, to a definite brownish-black color, with a hydrogen sulphide solution containing a known number of millimols of hydrogen sulphide per cubic centimeter.

Similar titrations were performed using a 2 per cent tryptone phosphate agar adjusted to the same H-ion concentration as the buffers employed in the previous experiment.

Some difficulty was encountered in the titration of the ferric ammonium citrate solutions. The iron compound reacts very slowly with hydrogen sulphide to form iron sulphide. Therefore, it was found necessary to run several series of these tubes and after the addition of a known amount of hydrogen sulphide the tubes were allowed to stand five or ten minutes before readings were made. This indicates that iron is not as sensitive to hydrogen sulphide as some other metals, for, in the case of bismuth, the formation of the sulphide was instantaneous.

In examining the data in table 1 the superiority of bismuth over iron is clearly shown. It should be noted that the amount of H_2S required to produce a brownish-black precipitate in the buffered solutions using bismuth as an indicator, was only 0.0048 millimol. When ferric ammonium citrate was used as an indicator the amount of H_2S varied from 0.02885 millimol at pH 8.0 and above to 0.101 millimol at pH 6.0. In the buffered menstruums bismuth proved to be 6 to 21 times more sensitive to H_2S than iron, depending upon the H-ion concentration. It is noteworthy that bismuth indicator is more sensitive to hydro-

gen sulphide in the presence of agar than in the buffered liquid menstruum. The ratio of sensitiveness of bismuth to iron varied from 8 to 1 at pH 9.0 to 24 to 1 at pH 6.0. Why the bismuth was more sensitive to hydrogen sulphide in an agar menstruum than in the buffer solutions cannot be stated. Inasmuch as the presence of hydrogen sulphide is generally determined in an agar medium this difference in the sensitiveness of bismuth over iron is of considerable importance, especially with organisms that produce such small quantities of hydrogen sulphide as to cause no color change with the ordinary indicators. The results of these experiments indicate that ferric ammonium citrate is not nearly

TABLE 1

Sensitivity of Bi and Fe as shown by the number of millimoles of H_2S required to show the presence of sulphides at various H^+ concentrations

pH	Buffered solutions			Nutrient agar		
	Bismuth	Iron	Sensitivity of Bi over Fe	Bismuth	Iron	Sensitivity of Bi over Fe
6.0	0.00481	0.101	21×	0.00240	0.0577	24×
6.5	0.00481	0.0481	10×	0.00240	0.0481	20×
7.0	0.00481	0.0481	10×	0.00240	0.0481	20×
7.5	0.00481	0.0481	10×	0.00240	0.0384	16×
8.0	0.00481	0.02885	6×	0.00240	0.02885	12×
8.5	0.00481	0.02885	6×	0.00240	0.02885	12×
9.0	0.00481	0.02885	6×	0.00240	0.0192	8×

as sensitive to hydrogen sulphide as bismuth. Ferric ammonium citrate reacts better in an alkaline menstruum while the reaction has no apparent effect upon the sensitivity of bismuth. The data are also presented graphically in figure 1.

It is believed that the reaction of ferric ammonium citrate with hydrogen sulphide at various hydrogen-ion concentrations should form a straight line but due to the difficulty in determining the end point this was not obtained.

The data presented here do not agree with the results reported by Zobell and Feltham. They found that ferrous iron was more sensitive than bismuth salts. Due to several factors it is, however, difficult to compare data. First, these investigators used bismuth carbonate which is an insoluble form of bismuth, and

therefore the sensitivity to sulphides was markedly reduced. Second, the medium containing the indicator was titrated with sodium sulphide which is a basic compound and, due to hydrolytic dissociation, would tend to make the medium more alkaline, in which case iron becomes more sensitive to the sulphide.

The results presented here not only agree with the theoretical consideration of these indicators, but definitely show the superiority of bismuth over iron in detecting hydrogen sulphide irrespective of the H-ion concentration.

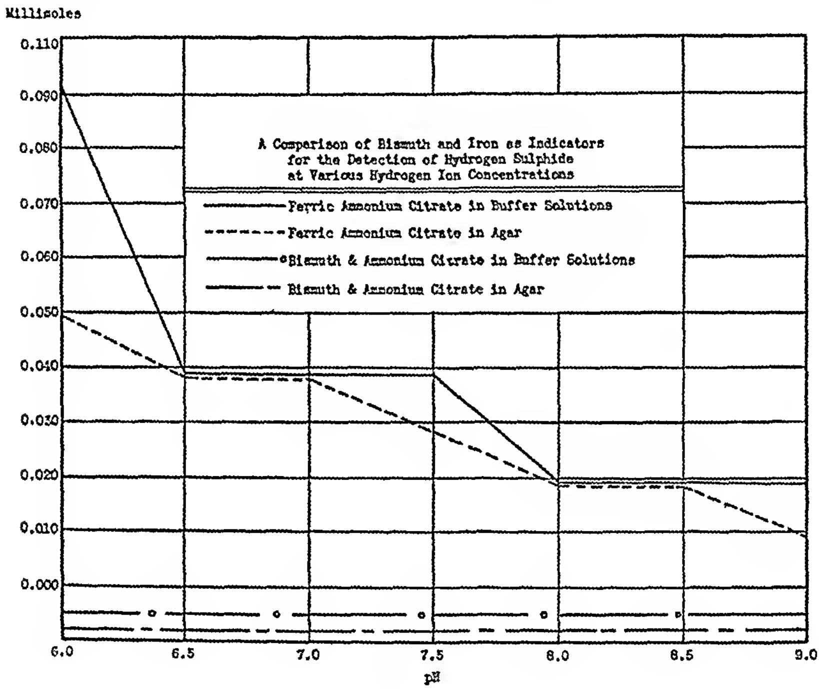


FIG. 1

PREPARATION OF A MEDIUM INCORPORATING BISMUTH

The second phase of work was to produce a medium incorporating a soluble bismuth compound as an indicator. This really developed into a problem of considerable magnitude. Soluble bismuth compounds are very active and react with many sub-

stances producing insoluble compounds under certain conditions. Experiments have been performed with over 650 media, some being of no value and others showing definite promise.

In the preparation of a medium containing a soluble bismuth compound one of the difficulties encountered was the formation of an insoluble precipitate. This difficulty was finally eliminated when it was discovered that Bacto tryptone and 0.5 per cent agar would not precipitate in the presence of bismuth, K_2HPO_4, glucose and sodium sulphite but that the use of any other peptone would produce a precipitate (Feldman and Hunter, 1935). After using a medium made from these ingredients for several months with good success the supply of Bacto tryptone, Bismuth citrate and glucose was exhausted. In obtaining new lots of these reagents difficulty with a precipitate developed as well as the reduction of the bismuth. In the past the reduction of bismuth by the glucose had never been encountered. Numerous experiments were performed to eliminate the precipitate in the medium and the reduction of bismuth. The reduction of bismuth was prevented by substituting mannitol, a non-reducing carbohydrate, but the elimination of the precipitate was more difficult.

During the experiments on the preparation of a medium which would not precipitate with bismuth it was discovered that Bacto-peptone iron could be used. The medium was prepared by adding 12 grams of the dehydrated peptone iron agar (one-third usual quantity) to 1000 cc. of distilled water, boiling until dissolved and then adding 10 cc. of 20 per cent Na_2SO_3 solution, 25 cc. bismuth liquor and 5 grams of mannitol. This medium contained such a small quantity of ferric ammonium citrate that it did not react with H_2S.

Further experiments brought out the fact that not all lots of Bacto-peptone iron could be used, as some would produce a precipitate. Many media were made, varying the ingredients, using different lots of tryptone, agar and other chemicals. The results of these experiments showed that the tryptone was the factor causing the precipitate. Some lots of tryptone did contain or did not contain a small quantity of material that reacted

with the bismuth causing the precipitate. Just what the factors are that cause this trouble has not as yet been determined.

When using the bismuth sulphite medium made with Bacto-peptone iron the criticism has been raised that ferric ammonium citrate may be responsible for the blackening. It has been definitely proven by numerous experiments that the blackening is due to bismuth sulphide and not iron sulphide. With organisms that produce only a small quantity of hydrogen sulphide a browning develops rather than the deep brownish-black precipitate. Another experiment was performed to prove that the iron was not responsible for any blackening and also that the bismuth was not reduced. In this case the medium was prepared omitting the bismuth liquor, inoculated with various organisms, incubated for 24 hours at 37°C. and then a small quantity of bismuth liquor was added. The results of this experiment check with those obtained when bismuth was present before inoculation.

In trying to overcome the development of a precipitate a series of experiments were performed varying the amount of each ingredient. The outcome of these experiments revealed that nearly as good results could be obtained by reducing the content of the bismuth to one-fifth (1/5) the original quantity. The color change of the medium would be brownish with small quantities of hydrogen sulphide and a deep brownish-black when larger amounts of sulphide were formed. Accordingly, the following medium was prepared using only 0.5 per cent of bismuth liquor with excellent results.

Medium I

Tryptone	7.0 grams
K_2HPO_4	0.3 gram
Agar	5.0 grams
Water	1000 cc.
Dissolve and filter, then add:	
Na_2SO_3 (20 per cent)	10 cc.
Bismuth liquor (3 per cent)	5 cc.
Mannitol	5 grams
Tube and sterilize at 15 pounds for 15 minutes.	

With most lots of tryptone, medium made according to this formula will not precipitate. It has, however, been noted that

certain lots of tryptone will precipitate. If this is the case it is advisable to use the formula for medium II. The Difco Laboratories have been able to reproduce medium I in a dehydrated form. This product has been thoroughly tested using several hundred cultures and the results are comparable to those of the original bismuth medium.

Medium II

The formula is the same as that for medium I except that 5.0 per cent of skimmed milk is added. No precipitate will form with any of the various lots of tryptone that have been tried. The medium is an opalescent color and the formation of hydrogen sulphide can easily be detected as the contrast in color is quite distinct.

Medium III

The medium is the same as No. II except that the agar content is increased to 1.5 per cent. The medium is slanted and both the slant and butt inoculated. The presence of H_2S on the surface of the slant will be noted by a brownish coloration with metallic sheen while in the butt of the tube the color will be a deeper brown or black. This medium has been used to prove that the brownish-black precipitate in media I and II was not reduced bismuth.

In comparing the bismuth medium with peptone iron it was found, as should be expected from the theoretical consideration of the indicators, that the bismuth medium would detect hydrogen sulphide formation in a larger percentage of cultures than would peptone iron. Organisms which are normally classified as non-hydrogen-sulphide-formers gave a definite reaction in the bismuth medium. In one series of experiments in which 80 stock cultures were inoculated into peptone iron and into bismuth medium it was found that after 48 hours 19 of the cultures or 23.7 per cent were positive in peptone iron and 46 or 57.5 per cent were positive in bismuth sulphite medium.

The comparison of results of some of the organisms in peptone iron and bismuth sulphite medium can be seen in table 2. Figure

2 shows the appearance of the different media with a few organisms. One thing to be noted is that *Salmonella paratyphi*, contrary to general opinion, does produce hydrogen sulphide as can be demonstrated by the use of bismuth indicator. This is not surprising when considering that bismuth is many times more

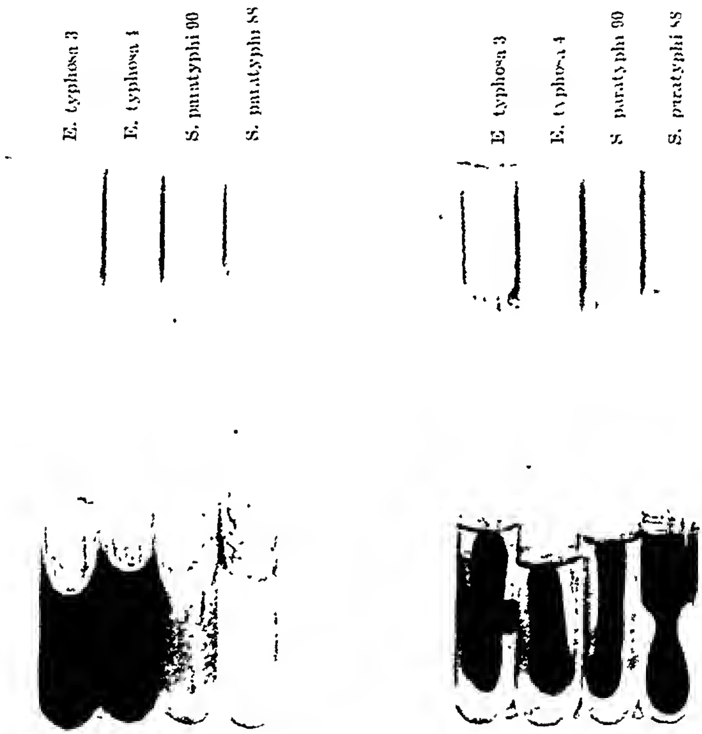
TABLE 2

H_2S reactions in various bismuth and iron media

ORGANISMS	BISMUTH-SULPHITE MEDIUM			PEPTONE IRON (DIFCO)		
	6-hour	24-hour	48-hour	6-hour	24-hour	48-hour
Esch. coli	+	+	++	–	–	–
Esch. communior "R"	–	–	–	–	–	–
Esch. communior	+	+++	+++	–	–	–
Esch coli Soule "S" 1	–	+	+++	–	–	–
Esch coli Soule "R" 2	+	+	+++	–	–	–
Aer. aerogenes	–	+	+	–	–	–
E. typhosa 6	–	–	+	–	–	–
E. typhosa 1	+++	++++	++++	–	+	+++
E. typhosa 2	–	++++	++++	–	+	+++
E. typhosa 3	–	++++	++++	–	+	++++
E. typhosa 4 ..	++	++++	++++	–	+	+++
S. paratyphi 90	+	++++	++++	–	–	–
S. paratyphi 89	–	+++	+++	–	–	+
S. paratyphi 88	–	++	++++	–	–	–
S. paratyphi 87	–	+	++	–	–	–
S. paratyphi 10	–	+	++	–	–	–
S. schottmulleri 21	–	++++	++++	+	+++	++++
S schottmulleri 20	++++	++++	++++	+	+++	++++
S. schottmulleri 93 ..	++++	++++	++++	+	+++	++++
S. schottmulleri 92	++++	++++	++++	+	+++	+++
S. schottmulleri 91	++++	++++	++++	–	+++	++++
Shig. paradysenteriae Flexner	+	+	++	–	–	–
Shig. paradysenteriae Flexner	+	+	++	–	–	–
Shig. dysenteriae	–	++	++	–	–	–

sensitive to hydrogen sulphide than are iron salts. The media have been criticized on account of being too sensitive and the differential value of hydrogen sulphide production is no longer valid in some cases. It is true that more organisms will probably indicate hydrogen sulphide production than by other methods but this should not eliminate its routine use. What the bac-

teriologist desires to know is whether an organism does or does not produce hydrogen sulphide regardless of past knowledge of the organisms. The differential value of the test may be decreased for the time being but future experience and investigation may prove it to be of distinct value. Until such time arrives it is suggested that hereafter the presence or absence of hydrogen



Bismuth-Sulphite Medium

Peptone Iron (Difco)

FIG. 2

sulphide be recorded according to medium or indicator used. It is not sufficient to record that an organism does or does not produce hydrogen sulphide.

SUMMARY AND CONCLUSION

1. Experiments to determine the sensitivity of ferric ammonium citrate and bismuth liquor for detecting hydrogen sulphide definitely show the superiority of bismuth over iron irrespective of

the pH. The most sensitive range of iron is in an alkaline menstruum while with bismuth the H-ion concentration has no effect.

2. After experimenting with more than 650 media, incorporating a soluble form of bismuth, 0.5 per cent bismuth liquor or 16 mgm. bismuth and ammonium citrate, Merck, U. S. P. IX, several formulae were developed which give good results. The variable factor is the tryptone, not all lots being of uniform composition. In case precipitation occurs with certain lots of tryptone the addition of 5 per cent of skim milk overcomes this difficulty.

3. The bismuth sulphite medium is far superior to any medium incorporating lead or iron for the detection of hydrogen sulphide in cultures. The results show that organisms producing a small quantity of hydrogen sulphide give negative results with iron while in the bismuth sulphite medium definite browning occurs.

4. Organisms which are usually stated in the literature to be negative hydrogen sulphide formers, such as *Salmonella paratyphi* and *Shigella dysenteriae*, give positive reactions with bismuth.

5. It is recommended that the indicator and method be stated when recording whether an organism does or does not form hydrogen sulphide. It is not sufficient to state that the organism is a negative or positive hydrogen sulphide producer.

REFERENCES

Bailey, S., and Lacey, G. R. 1927 A modification of the Kligler lead acetate medium. Jour. Bact., 13, 183-189.

Beckwith, T. D., and Moser, J. R. 1932 The reduction of sulphur containing compounds in the wood pulp and paper manufacture. Jour. Bact., 24, 43-52.

Burnet, E., and Wissenback, R. 1915 Valeur des renseignements fournis par la culture en gélose a l'acetate de plomb, pour la différenciation des bacillus typhique, paratyphique A et paratyphique B. Comparaison avec les résultats obtenus par l'agglutination, dans l'identification de 517 echantillons de bacilles typhique et paratyphiques. Compt. rend. de Soc. de Biol., 78, 565-568.

Feldman, M., and Hunter, C. A. 1935 An improved method for the detection of hydrogen sulphide produced by microorganisms. Proc. S. Dak. Acad. Science, 15, 41-45.

Hunter, C. A., Feldman, M., and Crecelius, H. G. 1937 Detection of hydrogen sulphide in cultures. Jour. Bact., 33, 31-32.

JORDAN, E. O., AND VICTORSON, R. 1917 Differentiation of the paratyphoid-enteritidis group. Jour. Inf. Dis., 21, 554–555.

KLIGLER, I. J. 1917 A simple medium for the differentiation of the members of the typhoid-paratyphoid group. Amer. Jour. Pub. Health, 7, 1042–1044.

LEVINE, M., VAUGHN, R., EPSTEIN, S. S., AND ANDERSON, D. Q. 1932 Some differential reactions in the colon-aerogenes group of bacteria. Proc. Soc. Exper. Biol. and Med., 29, 1022–1024.

MULSOW, F. W., AND PAINE, F. S. 1925 Hydrogen sulphide production by bacteria. Iowa Acad. Science, 32, 63–70.

MEYERS, JOHN 1920 The production of hydrogen sulphide by bacteria. Jour. Bact., 5, 231–252.

PACKECO, G., AND MELLO, J. T. 1932 Sur un procédé de determination de l'hydrogéne sulfure dans les cultures bactériennes. Compt. rend. de Soc. de Biol., 110, 131–132.

SCHUNK, I. V. 1924 Methods for class demonstration of hydrogen sulfide formation by bacteria. J. Elisha Mitchell Sci. Soc., 40, 107.

TANNER, F. W. 1917 Studies on the bacterial metabolism of sulfur. I. Formation of hydrogen sulfide from certain sulfur compounds under aerobic conditions. Jour. Bact., 2, 585–593.

THOMPSON, L. S. 1921 The group of hydrogen sulphide producing bacteria. Jour. Med. Res., 42, 383–389.

TILLEY, F. W. 1923 The relation between chemical composition of peptones and hydrogen sulfide production. Jour. Bact., 8, 287–295.

WILMET, M. M. 1927 Sur la sensibilité de quelques réactifs de l'hydrogéne sulfure gazeux. Compt. rend. de l'Acad. des Sciences, 184, 287.

WILSON, W. J. 1923 Reduction of sulphites by certain bacteria in media containing a fermentable carbohydrate and metallic salts. Jour. Hyg., 21, 392–398.

ZOBELL, C. E., AND FELTHAM, C. B. 1934 A comparison of lead and iron as detectors of hydrogen sulphide produced by bacteria. Jour. Bact., 28, 169–176.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, OCTOBER 26, 1937

DR. BERGEY AS I KNEW HIM. *Randle C. Rosenberger*, Jefferson Medical College, Philadelphia, Pa.

Our friend Dr. Bergey was born in Skippack, Montgomery County, Pennsylvania, in the vicinity of the school house in which Christopher Dock taught. A short distance away was another school house in which Dr. Bergey taught. How closely the life and works of Dr. Bergey resembled the life and works of Christopher Dock; who was virtuous in life, sweet in disposition, lovable in character and was America's pioneer writer on education, Pennsylvania's pioneer author, educator and a great leader. Dr. Bergey made friends because of his personality and scholarship. He gave his best, there was no half way. He was tolerant of others and understandable in all his trials, genuinely sincere in all his earthly dealings, rarely differed from any one, talked and let others talk, was at home in the presence of scholars and enjoyed the respect and confidence of his associates in an unusual degree. His integrity was unassailable. His greatest hobby was genealogy and his greatest tome was the compilation of the Bergey family, which was 1047 pages in length (without index) and comprised notes on 5759 individuals. His family life was ideal and his devoted wife was a bulwark in aiding him in his accomplishments.

THE RÔLE OF INHERITED NATURAL RESISTANCE TO TUBERCULOSIS. *Max B. Lurie*, The Henry Phipps Institute, Philadelphia, Pa.

By brother and sister mating of rabbit groups, families have been developed which exhibit varying inherited specific resistance to tuberculosis. This has been determined by natural respiratory exposure to artificially infected rabbits and by parenteral administration of standard quantities of tubercle bacilli. Irrespective of the mode of infection it was found that, by excluding all known environmental factors, the genetic constitution of the rabbit by itself may determine the type of disease developed. On first natural contact with the tubercle bacillus those hereditarily most susceptible develop a disease of short duration, characteristic of infantile tuberculosis in man, with miliary spread from a primary pulmonary-glandular complex. The animals of somewhat greater resistance develop a disease of longer duration, characteristic of the so-called childhood type, with caseous pneumonia, massive enlargement and caseation of the tracheobronchial nodes, and large nodular lesions of hematogenous origin in different parts of the body. The members of the most resistant family die on *first exposure* from a disease of long duration, which is characterized

by a localized, isolated, ulcerative pulmonary phthisis in which the tracheobronchial nodes and the rest of the body are not grossly affected and which is anatomically indistinguishable from adult pulmonary tuberculosis in man, which has hitherto been considered as resulting solely from reinfection.

The Nature of Inherited Natural Resistance to Tuberculosis. *Max B. Lurie*, The Henry Phipps Institute, Philadelphia, Pa.

By various procedures the following are found to be among the genetic factors that have thus far been tentatively shown to be related to the resistance to tuberculosis of the rabbit families described in the previous paper. (1) The primary sensitivity of the tissues on first contact with heat-killed tubercle bacilli. The greater the primary toxicity of the tubercle bacillus for the skin of an animal, the greater is the susceptibility of that animal to tuberculosis. (2) The acquired sensitivity of infection. All else being equal, a higher allergic sensitivity acquired during infection is associated with a greater resistance to tuberculosis, and, conversely, rabbits which develop the least sensitivity to tuberculin are often most susceptible to the disease. (3) The degree of allergy of infection is itself partly determined by the innate capacity for sensitization or responsiveness of the skin to substances in heat-killed tubercle bacilli. (4) The permeability of the skin to particulate matter. The greater the permeability, the greater the susceptibility to tuberculosis. None of these factors alone accounts for resistance, but their interaction and, doubtless, the collaboration of many other factors determine resistance. (5) An analogy has been found between the mode of reaction of artificially immunized and naturally highly resistant rabbits. In both instances the reaction to the tubercle bacillus is accelerated and abortive as compared with the reaction on first infection of rabbits of low inherited natural resistance.

A Study of the Agar Cup Plate Method. *Ruth E. Miller and S. Brandt Rose*, Woman's Medical College and The Philadelphia General Hospital, Philadelphia, Pa.

A study of the agar cup plate method showed that various factors must be taken into consideration when performing the test. These factors include: (1) the antiseptic under observation, (2) the species and strain of bacterial cell, (3) the size of the bacterial inoculum, (4) the protein content of the agar medium, (5) the pH of the medium, (6) the agar concentration, (7) the thickness of the agar medium and (8) the length of time which elapses between the preparation of the plate and the addition of the antiseptic. The information gained from the experimental data permitted a standardization of the agar cup plate method. When three antiseptics (phenol, mercury bichloride and crystal violet) were tested under controlled conditions, consistent results were obtained.

A Study of Mercury Antiseptics by the Agar Cup Plate Method. *S. Brandt Rose and Ruth E. Miller*, The Philadelphia General Hospital and Woman's Medical College, Philadelphia, Pa.

Using a standardized technic, four mercury antiseptics (mercury bichloride, mercurochrome, metaphen and merthiolate) were studied (1) in plain agar and (2) in horse-blood agar mix-

tures. The test organism was *Staphylococcus aureus*. All the mercury antiseptics showed a decreased zone of inhibition of bacterial growth as the blood concentration was increased. Parallel studies in blood broth and blood agar mixtures showed (1) that the agar *per se* had no appreciable effect on the zone size and (2) that in general a correlation could be established between the ordinary antiseptic dilution procedure and the agar cup plate method.

THE APPLICATION OF SINTERED (FRITTED) GLASS FILTERS TO BACTERIOLOGICAL WORK. *Harry E. Morton and E. J. Czarnetzky*, Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Until recently there were no filters available for bacteriological work which could be chemically cleaned as well as sterilized before use and which were adaptable to the filtration of small amounts of material. Sintered glass filters of No. 5 porosity presented these possibilities, if they could be relied upon to withhold bacteria. An article by the authors in the November issue of the JOURNAL OF BACTERIOLOGY set forth the tests performed to ascertain the reliability of these filters in withholding bacteria. Since the preparation of the above-mentioned manuscript the filters have been employed in research and teaching work for the filtration of solutions of soluble bacterial antigens, carbohydrates, etc. and found to be reliable in preventing the passage of bacteria. These filters can be used for the filtration of small amounts of material with very little loss. The cost of four dollars for the 3G5 auf 3 size is justified by the advantages which these filters possess.

CENTRAL PENNSYLVANIA BRANCH

KIRBY MEMORIAL HEALTH CENTER, WILKESBARRE, OCTOBER 30, 1937

THE GENERAL PRACTITIONER AND THE 1937 PNEUMONIA CAMPAIGN. *Angelo Luchi*, State Commission for the Study of Pneumonia Control.

Since there is no present sign that preventive medicine will make pneumonias as rare as typhoid fever, pneumonias remain a common medical emergency. In spite of recent advances in non-specific therapy the combined average death rate for the pneumonias is close to 25 per cent. By early and adequate specific treatment a reduction of the mortality to 5 per cent appears to be within the bounds of possibility. Unfortunately serum treatment is, and (unless it can be simplified) must remain, more suited to hospital than home conditions. The practitioner meets numerous difficulties: the laboratory aids are essential, but complicated and, usually, not available. Serum is costly, and the available types are few. Its administration requires time, skill and courage. Subsequent to the enumeration of the indications for serum therapy in the field, the following desiderata for better pneumonia control were advanced: (1) Determination of the cost of community provision of a complete pneumonia service; (2) Free distribution of antipneumococcic sera on the same basis as other biologicals, but limited to cases with a properly certified type in the first four days of illness; (3) Complete pneumonia laboratory service on a 24 hour basis; (4) A consultation and statistical service; (5) Education of practitioners, nurses and public.

PRODUCTION OF ANTIPNEUMOCOCCIC SERUM. *B. Scott Fritz*, Associate Director, The Gilliland Laboratories, Inc.

PNEUMONIA IN GENERAL. *H. S. Newcomer*, Chief, Medical Staff, Squibb Laboratories.

CHEMISTRY OF THE PNEUMOCOCCUS CAPSULE. *M. A. Farrell*, Division of Bacteriology, The Pennsylvania State College.

PNEUMOCOCCUS TYPING—SPUTUM AND BLOOD CULTURES—DEMONSTRATION. Lederle Laboratories, Inc.

CENTRAL NEW YORK STATE BRANCH

34TH SEMI-ANNUAL MEETING

COLLEGE OF MEDICINE, SYRACUSE UNIVERSITY, SYRACUSE, NEW YORK.

NOVEMBER 13, 1937

INCIDENCE OF GAS GANGRENE INFECTIONS IN NEW YORK STATE (EXCLUSIVE OF NEW YORK CITY). HOSPITAL REPORTS, 1932–1936 INCLUSIVE. *O. W. H. Mitchell*, College of Medicine, Syracuse University, Syracuse, New York.

CAN SEEDS BE STERILIZED? *Alvin W. Hofer and Herbert C. Hamilton*, New York State Agricultural Experiment Station, Geneva, New York.

In research work, it is usually necessary to sterilize all seeds that are to be inoculated with legume bacteria. This procedure raises a question concerning the efficiency of various sterilizing agents, and there is a problem as to the effect which might be produced upon the legume bacteria by any residual disinfectant remaining upon the seeds. A study of this problem was made by sterilizing lots of alfalfa and clover seed, drying them, and placing individual seeds upon Petri plates, to compare some of the more modern disinfectants with mercuric chloride (1:400 for 4 minutes) and chlorine water (2 per cent for one-half hour).

The number of viable bacteria remaining, as shown by the colonies that developed, was such as to suggest that complete sterilization of seeds is impossible. There was a level of contamination below which disinfectants were unable to reduce the number of bacteria. One disinfectant which had a phenol coefficient of 100 for pathogenic organisms proved worthless for the sterilization of seeds.

Mercuric chloride appeared to be variable in its effect, both in its ability to kill bacteria, and in its effect upon the nodule bacteria which were added later. So far chlorine water has proved as suitable as any agent for killing bacteria on the seeds, and it also possesses the advantage that it is easily washed from the seeds before inoculation.

A MICROSCOPIC METHOD FOR DIFFERENTIATION OF LIVING AND DEAD BACTERIA IN MILK. *Georges Knaysi and Mark Ford.* Cornell University, Ithaca, New York.

STANDARD AGAR COUNTS AS COMPARED WITH COUNTS ON IMPROVED AGARS AT 32°C. *M. W. Yale.* New York State Agricultural Experiment Station, Geneva, New York.

At the request of the Committee of the American Public Health Association on Standard Methods for the Examination of Dairy and Food

Products, Difco Laboratories supplied a dehydrated tryptone agar plating medium (0.5 per cent tryptone, 0.1 per cent glucose, 0.5 per cent skim milk and 1.5 per cent agar) to 91 laboratories, for comparison with standard nutrient agar. The milk was usually added by the laboratories previous to sterilization of the medium. A summary has been made of 43 reports by 56 laboratories on approximately 24,000 samples of dairy products.

Colonies were larger on the tryptone medium than on standard agar and easier to count. Counts from replicate 32°C. tryptone agar plates were less variable than those from 37°C. standard agar plates. With the exception of dry skim milk, incubation at 32°C. increased the count more than did the modified medium.

The increase in count in the case of the modified methods was in the majority of cases least for raw milk and progressively greater for Grade A pasteurized milk and Grade B pasteurized milk and greatest of all for pasteurized cream. There was practically no difference in the case of Certified raw milk. Wide distribution in the range of decreases and increases shows that adoption of the modified methods will not result in a constant percentage increase in count.

A Type of Bacterial Pigmentation Which Is Dependent upon the Presence of Light. *James A. Baker.* Veterinary College, Cornell University, Ithaca, New York.

Simultaneous Acceptance of Rhizobium by Phaseolus coccineus. *J. K. Wilson.* Department of Agronomy, Cornell University, Ithaca, New York.

Physiological Effects of Sodium Thiosulphate on Growth of Rhizobium Strains. *H. J. Webb.* Department of Agronomy, Cornell University, Ithaca, New York.

The growth-response of thirty-five strains of *Rhizobium* to sodium thiosulphate was determined using a photoelectric cell to measure relative growth. Six strains were originally isolated from alfalfa, (*Medicago sativa*), ten from amorpha, (*Amorpha fruticosa*), six from red clover, (*Trifolium pratense*), and thirteen from vetch, (*Vicia sp.*). The concentrations of sodium thiosulphate used were: 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, and 3.0 per cent. The control contained no sodium thiosulphate. In practically all cases 3.0 per cent sodium thiosulphate gave less growth than the control, and in many cases prevented growth entirely. The three lowest concentrations gave a stimulating effect in some cases but not in others. There was no uniform tendency with organisms representing any one plant-bacteria group. The variation between certain strains from one host-plant was as great as that between strains from various plant-bacteria groups.

The So-called Delayed Agglutination Reaction in Cattle Vaccinated against Bang's Disease. *Herbert L. Gilman.* Veterinary College, Cornell University, Ithaca, New York.

The plate (rapid) agglutination test is used extensively in the diagnosis of *Brucella* infection in cattle. The serum-antigen mixture is usually permitted to interact for eight minutes. Some workers, however, claim that with vaccinated animals the maximum titre is not reached until after fifteen minutes. Thus, plate agglutination tests were made on 542 vaccinated cattle, all under eighteen months of age. Readings were made at eight and fifteen minutes. The reading of 439

tests was the same at fifteen minutes as at eight minutes. A partial increase in titre, in a given dilution, was shown in 96 tests; of one plus in 4; of plus and partial in 3. No distinction was made between the degrees of partial agglutination at any one titre. If the readings on these samples were made regularly at fifteen minutes the interpretation would have been changed from negative to suspicious in eleven samples, and from suspicious to positive in an additional eleven samples. The changes were not considered highly significant. The work is being continued. The change in titre did not appear to depend on the time elapsing between vaccination and testing. A few samples showed an apparent lower reading at the end of fifteen minutes.

THE USE OF STOMACH CONTENTS IN PNEUMOCOCCUS TYPE DIFFERENTIATION. *O. D. Chapman.* College of Medicine, Syracuse University, Syracuse, New York.

INFECTIONS OF THE URINARY TRACT, DUE TO SHIGELLA PARADYSENTERIAE AND ALLIED SPECIES. *Erwin Neter.* Children's Hospital, Buffalo, New York.

The bacteriological findings in five patients (all female) with infections of the urinary tract, caused by members of the *Shigella* group, are presented.

In three of these cases, dysentery bacilli of the Hiss and Flexner types, respectively, were found in the urine. None of the patients gave history or evidence of intestinal dysentery, although two harbored the dysentery bacilli also in the intestinal tract. It may be suggested, therefore, that the infection of the urinary system originated in the intestines. In all cases, the serums contained agglutinins against the respective strains of high titer.

The other two cases of *Shigella* infections of the urinary tract were due to *Shigella alkalescens*, Andrewes. One patient developed agglutinins against her own strain (titer, 1:500), while the other failed to do so. Agglutination as well as agglutinin-absorption experiments, carried out with rabbit immune-serum and the serum of the above patient, revealed that the strains of *Shigella alkalescens* which were isolated contained at least three partial antigens: one, specific for *Shigella alkalescens*; the other two, common for different types of Flexner dysentery bacilli.

STUDIES ON THE RELATION OF SHIGELLA PARADYSENTERIAE NEUROTOXIN TO POLIOMYELITIS. *Andrew M. Zillig.* Department of Health Laboratories, Buffalo, New York.

Evidence is submitted to indicate that poliomyelitis and dysentery, apparently separate diseases may be due to the same etiological agent. Both occur at the same season of the year and prevail in the same localities. The symptoms of both vary from mild unrecognized cases to severe infections, gastric disturbance being an outstanding symptom, and under rather complex conditions a predominance of *Shigella paradysenteriae* endotoxins produce diarrhoea while neurotoxins produced by the same organism may be retained if constipation is a symptom, the nerve covering being injured by the inflammatory process. Conditions favor absorption of neurotoxin through branches to the nerve centers.

A study of the literature indicates that many investigators suspect the intestinal tract as the portal of entry. However, bacterial exotoxins are

known to be equally effective in the absence of organisms.

The colloidal gold curve for suitable specimens of dysentery stool filtrates was like that for poliomyelitic spinal fluid in the luetic zone.

Striking and rapid reactions were obtained in tests of blood-serum from typical cases of poliomyelitis with "O" Flexner and Hiss concentrated antigens, showing antigenic relationship and indicating the presence of toxic products of these organisms.

Neutralization and immunization tests with antiexotoxin, and serum therapy with polyvalent antidysentery sera containing expecially Flexner and Hiss antiexotoxins are suggested.

A Classification of Acid-fast Bacteria. *Ruth Gordon.* Veterinary College, Cornell University, Ithaca, New York.

Age Susceptibility in Johne's Disease. *W. A. Hagan.* Veterinary College, Cornell University, Ithaca, New York.

Bacteriological Findings in a Case of Human Infection from a Human Bite. *Basil G. Bibby and Margaret N. Barnes,* Department of Bacteriology, University of Rochester, School of Medicine and Dentistry, Rochester, New York.

The incompleteness of bacteriological data in the 5 fatal and 15 severe cases of infection from human bites, described in the literature, prompted us to record our findings in an additional fatal case. Three specimens each of blood and of pus from the area of injury were examined by smears, aerobic and anaerobic cultures. In addition, 40 human mouths were searched for anaerobic and microaerophilic cocci similar to those encountered in the pus and blood.

Smears from the pus showed Gram-positive cocci with occasional Gram-positive rods. Aerobic pus cultures revealed only atypical staphylococci. Anaerobic cultures showed three types of Gram-positive cocci, an irregular Gram-positive rod, a Gram-indifferent coccus and a Gram-negative coccus. *Fusiformis polymorphus* and a Gram-negative bacillus were present in smaller numbers.

Blood smears showed Gram-positive cocci two days before death. Aerobic blood cultures were negative, but anaerobic cultures always revealed a Gram-positive coccus. This organism resembled a streptococcus in morphology and colony form, but it fermented no sugars. After repeated subculture, it grew aerobically.

From the cultures from the 40 human mouths, one strain was grown for long enough to identify it definitely with the Gram-positive coccus obtained from the blood. Eight strains resembling the types found in the pus were recovered.

Reports in the literature emphasize the importance of anaerobic cocci in intractible gangrene and septicemia. Our studies indicate that such organisms exist in the human mouth.

Bacteriophage against Shigella paradysenteriae, Flexner, in an Outbreak of Dysentery in Macacus rhesus Monkeys. *Leslie A. Sandholzer,* Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, New York.

In the course of an outbreak of dysentery due to *Shigella paradysenteriae* (Flexner) in a colony of 62 *Macacus rhesus* monkeys, it was noted that freedom from the clinical disease

was associated with the presence either of antibodies or of bacteriophages against the invading organisms, while neither of these was demonstrable in animals developing the typical syndrome. Five of 7 sick animals recovered when given bacteriophage therapeutically. Freedom from symptoms in "carrier" monkeys was associated with the initial presence of a specific lytic agent in the intestine. In monkeys with initially high titers of agglutinins no evidence of infection or of the carrier state was found. Thirty-nine monkeys possessing neither antibodies nor bacteriophages were exposed to infection in the course of their association with sick or carrier monkeys in the animal quarters. These animals were given bacteriophage prophylactically and none of them developed the disease. When the colony was examined three months later, the treated monkeys showed agglutinins, but no bacteriophages. In the monkeys which had had bacteriophage initially, both a specific lytic agent and agglutinins were found. The initial status of the rest of the animals was unaltered by exposure to infection.

Relationships between Cultural Characteristics and Susceptibility to Bacteriophage in the Gram-negative Enteric Bacteria. *Leslie A. Sandholzer, Ralph P. Tittsler and George Packer Berry*, Department of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, New York.

No correlation was found between the susceptibility of *Escherichia, Aerobacter*, and *Shigella* strains to bacteriophages C13 and C16 (Burnet's pure strains) and any of the following characteristics: motility, fermentative ability, methyl-red and Voges-Proskauer reactions, utilization of citrate, or production of indol or hydrogen sulfide. Strains of a single species differed in lysability; some were lysed by both bacteriophages, some by only one, others by neither. Furthermore, many strains yielded, on dissociation, sub-strains (apparently identical in their biochemical reactions) which differed in susceptibility to these bacteriophages.

Following dissociation into R and S colonial types, a correlation was noted in most instances between susceptibility to C13 and colonial type. Thus, all sub-strains from 7 of the 8 parent-strains which yielded only R colonies were lysed, while all sub-strains from 24 of the 25 parent-strains which yielded only S colonies were not. The majority (26 of 33) of the R sub-strains from 13 dissociating parent-strains were lysed, while 28 of 39 S sub-strains were not. Most of the R types and about one-half of the S types were lysed by C16. Thus, C13 usually lyses R but not S strains, while C16 lyses most R and some S strains.

The differences in the susceptibility of bacterial strains to bacteriophage are explained best on the assumption of differences in the antigenic structure of the bacteria.

NORTH CENTRAL BRANCH

Iowa State College, Ames, Iowa, November 5–6, 1937

Respiration Functions of the Root Nodule Bacteria. *Robert Burris, D. W. Thorne and P. W. Wilson*, University of Wisconsin.

Methods are described for preparation of suspensions of "resting cells" of *Rhizobian trifolii*. The pH function of respiration shows a maximum in the

region of pH 6.0 about one unit lower than that for growth. The optimum temperature for respiration is 37°C., whereas that for growth is 28° to 30°C. When low concentrations of glucose are used as a substrate, only about two-thirds of it is respired, indicative that part of this substrate has been assimilated, probably as gum.

Light Intensity and the Nitrogen Hunger Period in the Soybean. *Orville Wyss and P. W. Wilson*, University of Wisconsin.

Previous observations on the inhibition of nitrogen fixation in nodulated soybeans by sunlight of high intensity were confirmed by further experiments during the past two summers. The inability of the soybean to initiate the nitrogen fixation process when exposed to light of high intensity was overcome both by shading and by addition of combined nitrogen. The results are consistent with the hypothesis that an excessive carbohydrate-nitrogen balance in the soybean plant inhibits nitrogen fixation.

The Ability of Rhizobia Isolated from Nodules of Wild Leguminosae to Fix Nitrogen in Symbiosis with Various Host Plants. *E. W. Ruf and W. B. Sarles*, University of Wisconsin.

A Method for Cultivating Root-Nodule Bacteria to Facilitate Staining of Their Flagella. *R. M. Stern and W. B. Sarles*, University of Wisconsin.

Studies on the Heat Resistance of Swiss Cheese Starter Cultures. *P. R. Elliker and W. C. Frazier*, University of Wisconsin.

Hydrogen as a Specific Inhibitor for Symbiotic Nitrogen Fixation. *Sylvan B. Lee, W. W. Umbreit and P. W. Wilson*, University of Wisconsin.

Experiments in which the *rates* of nitrogen fixation by red clover plants inoculated with *Rhizobium trifolii* have been determined indicate that hydrogen gas acts as a specific inhibitor for the symbiotic nitrogen fixation process. This confirms previous findings from this laboratory based on the *total nitrogen* fixed.

The Cultural Characteristics of Erysipelothrix Rhusiopathiae. *A. G. Karlson*, Iowa State College, Ames, Iowa.

A review of the literature indicates that there is much disagreement regarding the cultural and biochemic characteristics of *Erysipelothrix rhusiopathiae*. A study of 52 cultures revealed little variation between strains.

When isolated from tissue the microorganism is a short, slender, straight or curved rod 1 to 2 micra long. These form round, shiny colonies about 1 mm. in diameter.

When kept on agar, filaments 4 to 15 micra long may be found. These long thread-like forms form irregular, opaque colonies 2 to 4 mm. in diameter.

In gelatin stab cultures the growth radiates from the line of inoculation forming the test tube brush growth without liquefaction. Litmus milk may become slightly acid. A narrow green zone of hemolysis is seen around deep colonies in blood agar. Indol is not formed. The Voges-Proskauer reaction, methyl red, methylene-blue-reductase, and catalase tests are negative. Aesculin is not hydrolyzed. Hydrogen sulfide is readily formed.

No gas is formed on any fermentable media. Acid is produced readily from glucose, galactose, levulose, and lactose. A delayed reaction is seen in mannose and cellibiose. No reaction

is seen on arabinose, xylose, rhamnose, maltose, melibiose, sucrose, trehelose, raffinose, melezitose, dextrin, starch, inulin, amygdalin, salicin, glycerol, erythritol, adonitol, mannitol, sorbitol, dulcitol, or inositol. A reaction of pH 7.6 gives maximum growth at 37.5°C.

THE USE OF THE ROSSI-CHOLODNY AND BODENSTAUB METHODS IN THE STUDY OF TWO IOWA SOIL TYPES. *H. A. Wilson*, Iowa State College.

GROWTH FACTORS FOR THE BUTYL ALCOHOL BACTERIA. *R. W. Brown, H. G. Wood and C. H. Werkman*, Division of Bacteriology, Tuskegee Institute and Bacteriology Section, Iowa Agricultural Experiment Station.

An acidic ether-soluble extract of Difco-yeast-extract is essential for vigorous growth of the butyl alcohol organisms in a medium containing hydrolyzed casein 0.15 per cent, $(NH_4)_2SO_4$ 0.3 per cent, tryptophane 0.01 per cent, glucose 1.0 per cent and inorganic salts. An extract of 0.1 gram of yeast extract was used for 10 cc. of medium. There is a normal production of solvents in this medium. Growth was not obtained when 18 purified amino acids were substituted for the hydrolyzed casein. Apparently the hydrolyzed casein contains a second unknown factor, possibly an impurity (not B_1) or an essential amino acid other than the 16 tested.

THE DISSIMILATION OF MANNITOL BY THE PROPIONIC ACID BACTERIA. *Bruce R. Ford and C. H. Werkman*, Department of Bacteriology, Iowa State College, Ames.

The products of the mannitol fermentation by the propionic acid bacteria are *propionic, acetic, pyruvic, lactic*, and succinic acids and carbon dioxide. Qualitatively but not quantitatively the products are the same as in the glucose fermentation and show the same variation.

Since mannitol is a reduced compound each millimole fermented anaerobically is accompanied by the formation of at least one millimole of propionic acid. For every millimole of acetic and lactic acids formed, the amount of propionic acid above the minimum is decreased by an equivalent amount. The total of the oxidized compounds is decreased by a like amount. There exists a delicate balance among the neutral and oxidized products. If the carbon dioxide is high either the less oxidized compounds, pyruvic and succinic acids, or the neutral compounds, acetic and lactic acids, are low or the carbon is equally divided between the two groups. If the carbon dioxide is low the carbon is recovered in higher yields of neutral or other oxidized compounds, or both. Acetic acid is relatively constant. Lactic acid may be present or absent. Pyruvic and succinic acids may be absent, present equally, or either may predominate or be absent.

ERRATUM

In the article by Hunter and Crecelius on "Hydrogen Sulphide Studies," the legends for the two halves of Fig. 2 on page 194 were unfortunately reversed. The figure on the left hand is "Peptone Iron (Difco)" and the one on the right "Bismuth-Sulphite Medium."

THE CHARACTERISTICS OF ANTIPNEUMOCOCCUS SERA PRODUCED BY VARIOUS ANIMAL SPECIES[1]

F. L. HORSFALL, JR.

The Council of the Society of American Bacteriologists has requested that a report be made of the work for which the award of the Eli Lilly and Company medal was made. I am happy to comply with that request and will attempt to describe briefly and in general terms the studies upon which we have been engaged for the past three years at the Hospital of the Rockefeller Institute. All of the work has been carried on in continuous association with Dr. Kenneth Goodner. The various problems have been attacked simultaneously from two separate lines of approach and the results have been the outcome of the constant collaboration of both members of the team.

It is peculiar fact that various animal species respond in quite different manners to the same antigenic stimulus. For example, the intravenous injection of killed Type I pneumococci into either horses or rabbits results eventually in the production of antibacterial substances by both species. Although these substances give reactions which are dominantly type specific, there are many significant differences between their various properties.

Throughout these investigations we have been interested chiefly in the numerous differences between type specific antipneumococcus sera produced by various animal species. All of the studies have been directed toward these antibacterial sera and in the discussion which follows it will be understood that antipneumococcus serum is meant whenever reference is made simply to antiserum.

Although a large portion of the studies have dealt with prop-

[1] Address delivered before the Society of American Bacteriologists, December 30, 1937, by the recipient of the Eli Lilly and Company Research Award in Bacteriology and Immunology.

erties of antisera which may be of only theoretical interest, the motivating consideration behind them all has been the development of a therapeutic antiserum even more effective than antisera now generally used in the treatment of pneumococcus pneumonia.

In the time which is allowed me it will not be possible to give more than a sketchy summary of the various laboratory studies which have been carried on, nor will it be feasible to do more than briefly suggest some of the numerous implications of the experimental results. Since these have led us to try rabbit antiserum in the treatment of human pneumonia, a short sketch of the experimental basis for the clinical trials seems desirable.

For a number of years it has been known that the injection of the same antigen into horses and rabbits produced antisera which possessed a number of directly opposed characteristics. Schiemann and Casper showed that specific precipitates from horse antiserum were quite different microscopically from similar precipitates of rabbit antiserum. Zinsser and Parker found that capsular polysaccharides and horse antiserum do not fix complement, although complement is bound by the polysaccharide and rabbit antiserum. Avery and Tillett demonstrated that horse antiserum does not sensitize guinea pigs to the capsular polysaccharide; while, after the injection of rabbit antiserum, these animals can be shocked anaphylactically with the polysaccharide. Both Avery and Felton had repeatedly shown that the antibody of horse antiserum was precipitated with the globulin, by dilution of the antiserum with water. With rabbit antiserum, precipitation does not occur on similar dilution. Goodner had noted that horse antiserum was less effective than rabbit antiserum in the therapy of experimental dermal pneumococcus infections. Heidelberger and Kendall had demonstrated that rabbit antisera possessed a narrower immunological specificity than did horse antisera.

No entirely adequate explanation for these differences in the antisera of the two species was at hand. In attempting to define the underlying basis for these divergent properties numer-

ous other differences were encountered and it was found, also, that in antisera produced by some thirteen separate mammalian species similar differences existed. Among the antisera of these species there seemed to be two groups, and only within each group were the reactions of the antisera identical. Between the two groups the various reactions of the antisera had many different characteristics. The antiserum of the horse can be taken as the prototype of the first group and that of the rabbit as the prototype of the second group.

As an indication of one of the striking differences between the antisera of these two species let us consider first the effects of extraction of lipids from their respective antisera. In both cases, after thorough extraction of lipids, the antisera fail to exhibit the characteristic reactions of specific agglutination and precipitation although protective potency is unaltered. In the case of antisera of the first group, of which the horse is the prototype, the addition of very small amounts of lecithin to the extracted antiserum restores the specific *in vitro* reactions. However, in the antisera of the second group, of which the rabbit is the prototype, cephalin is essential for the restoration of specific *in vitro* reactions to the extracted antiserum.

In most of the work the characteristics of horse and rabbit antisera have been studied, partly because of the availability of these two antisera but chiefly because their content of antibody is higher than is encountered in the antisera of any of the other species studied. These two antisera have been examined in as many ways and by means of as many techniques as was possible.

In all, some thirty-five distinctly different properties have been encountered in the type specific antisera of the rabbit and the horse. Among these some of the more important are the following:

Horse antiserum when used in more than a definite optimum amount causes a complete inhibition of protective action, a phenomenon which has been termed the protective prozone. Rabbit antiserum does not cause an inhibition in protective effect, no matter how much is injected. The addition of purified cephalin to optimum protective quantities of horse antiserum

causes a marked inhibition in protective action; but the addition of purified lipids to rabbit antiserum has no effect upon its protective potency. Specific precipitates from horse antiserum prepared by the method of Heidelberger and Kendall absorb cephalin from the reacting mixture whereas similar precipitates from rabbit antiserum absorb lecithin. The presence of lecithin is essential to specific agglutination and precipitation by horse antiserum, while the presence of cephalin is essential to these *in vitro* reactions in the case of rabbit antiserum. On this basis and because it has been found that lecithin is present in immunologically pure preparations of horse antibody globulin, it has been suggested that the type-specific antibody of antipneumococcus serum is a globulin-phosphatide complex, and that the character of the phosphatide is dependent upon the species which produced the antibody. By means of ultrafiltration experiments, conducted with Dr. Johannes Bauer, it was found that the smallest antibody globulin from horse antiserum is approximately four times larger than the smallest antibody globulin in rabbit antiserum. Heidelberger and Pedersen have found similar differences in the size of these antibodies by means of the ultracentrifuge. Various fractions of antibody globulin from horse antiserum may vary as much as seven-fold in protective potency, whereas the various fractions of antibody globulin from rabbit antiserum are approximately identical in this respect. One gram of the total antibody globulin from horse antiserum possesses, on the average, only one-half the protective potency of an equal quantity of antibody globulin from rabbit antiserum.

During the course of various experiments directed towards the study of the differences in the antisera of these two species, it was found that in standard mouse-protection tests rabbit antisera possessed, on the average, about three times greater protective potency than did horse antisera. It was also found that rabbits are capable of producing highly potent antisera in a much shorter period of time than are horses. When this point was directly examined it developed that the hyper-immunization of rabbits required but one-tenth the time usually required for horses.

Because of the large number of differences found experimentally between the antisera of the horse and the rabbit it seemed probable, from both theoretical and practical considerations, that rabbit antiserum would be more effective than horse antiserum as a therapeutic agent in pneumococcus pneumonia. Consequently, after suitable precautions had been taken and adequate amounts had been prepared, rabbit antiserum was given clinical trial. In all of the clinical studies Dr. Colin MacLeod has had an equal share. Early in the course of the clinical studies it developed that untreated rabbit antisera frequently produced severe chill reactions after intravenous administration to human beings. It was found that heating the antiserum to 56°C. for thirty minutes, and subsequently absorbing it with sterile kaolin, considerably reduced the content of chill-producing substances. Later it developed that the quantity of chill-producing substances in rabbit antisera could be quite accurately determined by the intravenous injection of 2.0 cc. into normal rabbits. The extent of the thermal reaction caused in normal rabbits paralleled quite closely the degree of chill reaction produced in human beings. Antisera which have caused a mean elevation in temperature of less than 1.2°F. in three normal rabbits have not caused chill reactions when given intravenously to patients with pneumococcus pneumonia. It has been found also that rabbit antisera can be given considerably more rapidly than horse antisera, and in many instances it has been possible to give an adequate therapeutic dose in a single intravenous injection. To date, sixty-seven patients with pneumococcus pneumonia have been treated with type-specific rabbit antiserum. Thirteen patients had Type III pneumonia, and in this group no definite evidence was obtained that the antiserum had been effective. Fifty-four patients had either Type I, II, V, VI, VII, VIII, XIV, or Type XVIII pneumonia. In this group the average interval from the beginning of rabbit serum therapy until the acute signs of the disease had disappeared was but twenty-seven hours. In eighteen patients who were given an adequate therapeutic quantity of antisera in a single injection the average interval from the administration of antiserum until

the completion of crisis was but $7\frac{1}{2}$ hours. Pneumococcus bacteremia occurred in thirty-three per cent of cases, multilobar consolidation occurred in thirty-one per cent, and infected pleural exudates in thirteen per cent. Type-specific antibody of rabbit origin became demonstrable in the infected pleural exudates of three patients after the intravenous injection of antiserum. In these three instances the pneumococci subsequently disappeared from the pleural exudates. In this group of fifty-four cases there have been but two deaths, a mortality rate of 3.7 per cent.

Although the use of rabbit antiserum as a therapeutic agent in pneumococcus pneumonia is still in the experimental stage, the results obtained so far seem to have justified the initial premise which was based upon laboratory studies. It is of considerable interest that studies which centered largely around theoretical problems have resulted in the development of therapeutic procedures which appear to have practical significance in the treatment of the highly fatal infectious disease, pneumococcus pneumonia.

THE ELECTROPHORETIC MIGRATION VELOCITY OF ESCHERICHIA COLI AFTER CULTIVATION ON MEDIA OF VARYING COMPOSITION[1,2]

I. Observations Following Changes in Organic Constituents

RALPH P. TITTSLER AND GEORGE PACKER BERRY

Department of Bacteriology, University of Rochester, School of Medicine and Dentistry, Rochester, New York

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Numerous investigators, especially Northrop and DeKruif (1922) and Falk and his colleagues (1925, 1926 and 1928), have found that changes in bacteria which may be recognized by alterations in virulence, toxigenicity, agglutinability, etc. are accompanied by measurable differences in the electrophoretic migration velocity of the organisms. It is well known that such changes in bacteria, i.e., variations or dissociations, occur spontaneously in nature and that they can be induced under experimental conditions, especially by the incorporation of certain ingredients in the culture medium. These facts have suggested that changes in the physico-chemical structure of bacteria arising during dissociation or variation might not only be detectable, but also susceptible of some analysis by the method of electrophoresis. Accordingly, electrophoretic methods have been employed by us to study the effects of variation in the composition of the culture medium upon the structure and behavior of bacteria. The part of the investigation reported herewith was

[1] Based on portions of a thesis presented by Ralph P. Tittsler, June 1, 1933, to the Faculty of the Graduate School of the University of Rochester in partial fulfillment of the requirements for the degree, Doctor of Philosophy.

[2] This work was supported in part by contributions from the General Education Board Research Fund and the Department of Biology, College of Arts and Science, University of Rochester.

undertaken to ascertain specifically the effect of differences in the organic composition of the culture medium upon the electrophoretic potential of *Escherichia coli.*

MATERIALS AND METHODS

Culture media. For the present investigation 35 different culture media were used. In their preparation the ingredients were made to vary both qualitatively and quantitatively. The greater emphasis was placed on the latter type of variation, since in this respect each medium differed from all others, while qualitatively there were 5 important differences. The composition of each medium is recorded in table 1, where each different combination is given a key number for convenience of designation in this report.

The agar, peptone and meat extract employed were Difco products. Fresh, defibrinated rabbit-blood was used in medium 35. Only distilled water was employed. All media were dispensed in Pyrex glassware and with the exception of medium 35 were sterilized in the autoclave at from 15 to 18 pounds pressure for 20 minutes. To prevent evaporation, the media were stored in an icebox. A few hours before inoculation, however, they were placed at 37°C. to raise the temperature to that used for incubation.

Test organism. The strain of *Escherichia coli* used in this study was isolated from human feces in 1927 and has been maintained since then by cultivation on nutrient agar. It was used by Lisse and Tittsler (1932), Dozois, Tittsler, Lisse and Davey (1932) and Pearce, Lisse and Tittsler (1935) for various studies in electrophoresis. On nutrient agar, it produced the "S" type of growth.

Inoculation and incubation of cultures. Since the major purpose of this study was to determine whether or not the electrophoretic behavior of *Escherichia coli* was altered by continuous cultivation on media of various composition, 10 serial transfers at 24-hour intervals were made on each medium, except 31 and 35. A uniform starting point for each experiment with each of the media studied was secured by initiating each experiment from a "stock"

TABLE 1

Individual formulae of culture media

MEDIUM NUMBER	AGAR	PEPTONE	MEAT EXTRACT	pH
	per cent	*per cent*	*per cent*	
1	1.0	0.5	0.3	6.8
2	2.0	0.5	0.3	6.8
3	3.0	0.5	0.3	6.8
4	4.0	0.5	0.3	6.8
5	5.0	0.5	0.3	6.8
6	2.0		0.3	6.7
7	2.0	0.125	0.3	6.8
8	2.0	0.25	0.3	6.9
9	2.0	0.5	0.3	6.9
10	2.0	1.0	0.3	6.9
11	2.0	2.0	0.3	7.0
12	2.0	3.0	0.3	6.9
13	2.0	5.0	0.3	7.0
14	2.0	0.5		6.9
15	2.0	0.5	0.10	6.9
16	2.0	0.5	0.25	6.8
17	2.0	0.5	0.5	6.8
18	2.0	0.5	1.0	6.8
19	2.0	0.5	2.0	6.7
20	2.0		0.10	6.6
21	2.0		0.25	6.6
22	2.0		0.5	6.6
23	2.0		1.0	6.6
24	2.0		2.0	6.5
25	2.0	0.25		6.9
26	2.0	0.5		6.9
27	2.0	1.0		6.9
28	2.0	2.0		6.9
29	2.0	3.0		6.9
30	2.0	5.0		6.9
31		0.5	0.3	6.8
32	1.5	0.5	0.3	6.8
33	4.5	1.5	0.9	6.7
34*	4.5	1.5	0.9	6.7
35†	2.0	1.0		7.0

* This medium also contained 1.5 per cent of glucose.

† This medium also contained 5.0 per cent of rabbit-blood and 0.8 per cent of sodium chloride, added to prevent hemolysis.

series of cultures, carried continuously on standard nutrient agar (medium 32) with transfers every 24 hours. To provide the actual bacteria for observation in the electrophoresis cell, transfers from the "stock" culture and from the second, fourth, seventh and ninth serial transfers on each medium were made to a slant of the particular medium under investigation and after incubation for from 12 to 15 hours the cells were suspended as described below. Thus, bacteria from the first, third, fifth, eighth and tenth serial passages were used for electrophoretic measurements. All incubations were carried out at 37°C. The period of incubation for source cultures was chosen arbitrarily because seven sets of preliminary experiments showed no differences in the electrophoretic migration velocities of bacteria derived from cultures which had been incubated for 10, 12, 16, 20 and 24 hours.

Preparation of bacterial suspensions for electrophoresis. The bacteria were washed from the surface of the agar slant with a small amount of double-distilled water, after which the suspension was diluted with double-distilled water to a volume of approximately 40 cc. and centrifuged to sediment the cells. The clear supernatant was discarded and the bacteria were resuspended in double-distilled water. This procedure was repeated twice to wash the organisms three times. The sedimented mass of bacteria was shaken thoroughly after each centrifugalization to insure a uniform washing of the individual cells.

After the third washing the bacteria were again suspended in double-distilled water, the suspension was filtered through absorbent cotton and diluted to an arbitrarily chosen turbidity. The suspension was then ready for electrophoretic determinations. Not more than 8 hours elapsed between the final preparation of the suspension and its use in the electrophoretic apparatus.

The procedure of thrice washing the bacteria was based upon the results (table 2) of a comparative study of the electrophoretic migration velocity of twice and thrice washed cells. The velocity of the thrice-washed bacteria averaged 20 per cent greater than that of the twice-washed, i.e., the migration time in seconds was less. The greatest difference between any two of the thrice-

washed suspensions was only 0.34 second or 5 per cent, while the value was 1.05 seconds or 13 per cent with twice-washed preparations. Thus, the third washing increased the uniformity of migration velocity. When combined with previous experiences, these results established the necessity of using thrice-washed cells, if accurate data concerning the intrinsic nature of the bacteria were to be obtained.

Glassware. Because the electrophoretic migration velocity of bacteria is greatly affected even by traces of electrolytes in the suspending medium, special attention was given to the quality

TABLE 2

Electrophoretic migration time in seconds of twice and of thrice washed bacteria from cultures grown on various concentrations of agar

AGAR	EXPERIMENT 1		EXPERIMENT 2		EXPERIMENT 3	
	Times washed					
	2	3	2	3	2	3
per cent						
1	8.03	6.93	9.04	7.06	8.52	6.98
2	8.14	7.04	8.52	7.22	8.33	6.89
3	8.01	6.88	8.80	7.02	8.69	7.07
5	7.99	6.90	8.57	7.09	8.55	7.13
Average....	8.04	6.91	8.74	7.10	8.52	7.02
Percentage change...		+16.35		+23.24		+21.36

and cleanliness of all glassware used in the preparation and storage of both distilled water and bacterial suspensions. Chemically clean Pyrex glassware, therefore, was employed exclusively.

Electrophoresis apparatus. Mudd's modification of the Northrop-Kunitz microscopic electrophoresis apparatus was used. At the end of each day's work, the observation cell was removed and treated to render it chemically clean. Prior to making any determinations and after recording each set of determinations, the apparatus was rinsed thoroughly with double-distilled water.

The electrophoretic migration velocities were determined at depths of five-twelfths, six-twelfths, and seven-twelfths of the

inside vertical diameter of the electrophoresis cell. At each specific level, ten measurements were made, reversing the polarity of the electrodes after each fifth determination. Thus, 30 measurements in all were made. The cell was then refilled and a second set of measurements made. The two sets were averaged and the averages compared. Provided that the difference between them was not greater than 4 per cent, the results were accepted. When a greater difference was encountered, a rare occurrence, further measurements were made. The values reported are the average number of seconds required for the bacteria to be moved a distance of 340 microns under the influence of a potential gradient of 3.5 volts per centimeter.

Experimental error. During this investigation and in previous studies (Tittsler and Lisse, 1928; Lisse and Tittsler, 1932; Tittsler, Lisse and Ferguson, 1932) it was found that the determinations made on one day might differ by as much as 10 per cent from those obtained on another day, despite the fact that the bacteria under investigation had been grown on the same lot of culture medium and had been handled as far as possible in an identical manner. In most instances, however, the differences observed from day to day were much smaller. When a series of determinations was made on the same day on an individual sample, on the other hand, the range of variations seldom exceeded 4 per cent. In general, this is in accord with the experience of other investigators. Differences as great as 10 per cent, therefore, are in certain instances within the range of the experimental error.

RESULTS

The electrophoretic migration velocity of *Escherichia coli*, when measured under strictly uniform conditions, was found to be constant regardless of qualitative or quantitative differences in the composition of the culture medium. It was also constant during serial cultivation on the various media. Obviously, the migration velocity was not exactly the same for all of the 200 suspensions of bacteria. The differences, however, were so small that they must be attributed to experimental error. Further-

more, they did not parallel either the differences in the culture media or the progress of the serial cultivation.

Since the accumulated data are too voluminous to give in detail, and because the migration velocity was constant, only the summarized results of one typical experiment are presented. In this instance, the culture media, nos. 6 to 13 inclusive, were made to vary in their content of peptone. The results of electrophoretic migration measurements are recorded in table 3. It is evident that the migration velocity of the bacteria was not influenced by differences in the concentration of peptone. The greatest difference between the migration time of bacteria from the first and tenth serial transfers on the same medium (0.125 per cent peptone) was a decrease of 0.61 second or 8.7 per cent and the average difference between all of the first and tenth transfers was 0.44 second or 6.4 per cent. The greatest difference occurred between the first and third serial transfers, but it was only 0.29 second or 4.2 per cent. Differences of this magnitude have no significance for they do not exceed the experimental error.

TABLE 3

Electrophoretic migration time in seconds of bacteria from serial transfers on media containing various concentrations of peptone

PEPTONE*	SERIAL TRANSFER NUMBER					AVERAGE
	1	3	5	8	10	
per cent						
0.00	6.85	6.73	6.68	6.47	6.39	6.62
0.125	6.93	6.49	6.54	6.59	6.32	6.57
0.25	6.93	6.61	6.66	6.42	6.48	6.62
0.50	6.75	6.77	6.44	6.48	6.56	6.60
1.0	6.89	6.61	6.63	6.43	6.29	6.57
2.0	6.73	6.11	6.62	6.49		6.49
3.0	6.76	6.40	6.57	6.45	6.25	6.49
5.0	6.78	6.62	6.48	6.57	6.45	6.58
Average..	6.83	6.54	6.58	6.49	6.39	6.57

* Each of these media, nos. 6 to 13 inclusive (table 1), contained 0.3 per cent of meat extract and 2 per cent of agar.

Similar results were obtained with the other media listed in table 1. There was no evidence that the migration velocity of the bacteria was influenced by any of the qualitative or quantitative differences in the culture media. The actual differences between the velocities of the bacteria from either the various media or from the different serial transfers were usually less than those in the experiment just cited.

The results of preliminary experiments showed that it was necessary to exercise extreme care in the preparation of bacterial suspensions to be employed for electrophoretic determinations if reliable results were to be expected. The migration velocity was affected enormously by slight variations in the pH of the distilled water in which the bacteria were suspended and by the presence of traces of electrolytes. It was imperative, therefore, to employ only double-distilled water for washing and suspending the bacteria and to use only chemically clean glassware.

Electrophoretic measurements were made on more than 100 suspensions of bacteria before and after storage for from 4 to 8 hours at room temperature and the results showed that the migration velocity was not altered during that period.

DISCUSSION

Since the purpose of this study was to investigate by the electrophoretic method the effects of differences in the organic composition of the culture medium on the physico-chemical structure of bacteria, it was necessary to eliminate factors other than changes in the bacteria themselves which influence the electrophoretic migration velocity and to determine the rate of migration under strictly uniform conditions. The presence of even traces of electrolytes in the suspending medium are known to produce enormous changes in the migration velocity (Northrop and De-Kruif, 1922; Winslow, et al., 1923 and 1926). Thus, it was imperative to wash the bacteria until they were free of all extraneous electrolytes and metabolic products. As described previously, only thrice-washed bacteria, double-distilled water, chemically clean glassware, and a standardized procedure were employed. Under these conditions significant differences in the

observed electrophoretic migration velocity indicate an alteration of the physico-chemical constitution of the cells and, conversely, the maintenance of a fixed velocity is evidence that the bacteria have not changed.

A pertinent question is whether or not every change in a bacterium must necessarily be reflected by an altered electrophoretic migration velocity or, conversely, whether or not every change in the electrophoretic migration velocity must be an indication of an alteration in the physico-chemical composition of the cell. It is conceivable that certain alterations in the cell might occur without changing its electrophoretic potential. Such changes, however, would have to be independent of the cell wall and could not involve variations in ionic diffusion. Apart from such unknown, but theoretically possible changes, it follows that constancy of the electrophoretic migration velocity of bacteria is strong evidence, yet not necessarily proof, that the cells have not changed.

On the basis that a constant electrophoretic velocity of cells indicates no change in the bacteria, *Escherichia coli* grown on a multiplicity of culture media was able to propagate without change. These results are interesting because of their bearing on the subject of bacterial dissociation and variation, and suggest that it would be profitable to extend this type of investigation to embrace studies of the effect on the electrophoretic potential of introducing into the culture medium substances, such as sodium, calcium and lithium chlorides, which are known to cause bacterial dissociation. The results of such observations will be presented in a forthcoming paper.

SUMMARY

The electrophoretic velocity of *Escherichia coli*, when measured under strictly uniform conditions, remained constant during serial cultivation on a wide variety of culture media differing qualitatively and quantitatively in their organic ingredients.

The constant electrophoretic velocity of the bacteria is considered to constitute strong evidence, but not necessarily proof, that the physico-chemical composition of the cells was not changed by variations in the culture medium.

REFERENCES

DOZOIS, K. P., TITTSLER, R. P., LISSE, M. W., AND DAVEY, W. P. 1932 The effect of "low voltage" x-rays on the electrophoretic migration velocity, viability and pH of *Escherichia coli* suspensions. Jour. Bact., 24, 123–132.

FALK, I. S. 1932 "A Theory of Microbic Virulence," Chapter 12 in The Newer Knowledge of Bacteriology and Immunology, Jordan and Falk. University of Chicago Press, Chicago.

FALK, I. S., AND JACOBSON, M. A. 1925 Studies on respiratory diseases. XXIV. Electrophoretic potential, virulence and serum agglutination with single cell cultures of variants of Type I pneumococci. Jour. Inf. Dis., 37, 507–513.

FALK, I. S., AND JACOBSON, M. A. 1926 Studies on respiratory diseases. XXVI. Electrophoretic potential, acid and serum agglutination of pneumococci. Jour. Inf. Dis., 38, 182–187.

JENSEN, L. B., AND FALK, I. S. 1928 Electrophoresis of diphtheria bacilli. I. Some relations between the virulence, toxigenicity and electrophoretic mobilities of diphtheria bacilli. Jour. Bact., 15, 367–411.

LISSE, M. W., AND TITTSLER, R. P. 1932 The effect of irradiation on the electrophoretic velocity, viability, agglutinability, lysis, and pH of *Escherichia coli*. Penna. Agri. Expt. Sta. Tech. Bull. 276.

NORTHROP, J. H., AND DEKRUIF, P. H. 1922 The stability of bacterial suspensions. II. The agglutination of the bacillus of rabbit septicemia and of *Bacillus typhosus* by electrolytes. Jour. Gen. Physiol., 4, 639–655.

PEARCE, G. W., LISSE, M. W., AND TITTSLER, R. P. 1935 Influence of electrolytes added to growth medium on electrophoretic potential of *Escherichia coli*. Proc. Soc. Exp. Biol. and Med., 32, 1572–1573.

TITTSLER, R. P., AND LISSE, M. W. 1928 The relation between electrical charge and the agglutinating ability of *Salmonella pullorum*. Jour. Bact., 15, 105–116.

TITTSLER, R. P., LISSE, M. W., AND FERGUSON, R. L. 1932 The electrophoretic potential of *Rhizobium meliloti*. Jour. Bact., 23, 481–489.

WINSLOW, C.-E. A., FALK, I. S., AND CAULFIELD, M. F. 1923 Electrophoresis of bacteria as influenced by hydrogen ion concentration and the presence of sodium and calcium salts. Jour. Gen. Physiol., 6, 177–200.

WINSLOW, C.-E. A., AND FLEESON, E. H. 1926 The influence of electrolytes upon the electrophoretic migration of bacteria and of yeast cells. Jour. Gen. Physiol., 8, 195–214.

SOME FUNDAMENTAL INVESTIGATIONS ON THE RESISTANCE OF TUBERCLE BACILLI

H. J. CORPER AND MAURICE L. COHN

Research Department, National Jewish Hospital, Denver, Colorado

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The tubercle bacillus possesses certain individual characteristics which differentiate it from other microörganisms. This organism shows a resistance to certain physical and chemical agents similar to that noted for spore-forming types of fungi. Yet, the existence of spores for tubercle bacilli has been a matter of suggestion rather than fact because of the difficulties in demonstrating such a definition. It is conceivable that if spore forms existed for tubercle bacilli under adverse conditions, this more resistant type would develop a variable protection against physical and chemical agents in proportion to the nature and number of forms present.

The addition of lipin and waxy-like materials to the bacillary body by enhanced anabolism (when growing under favorable nutrient conditions) might result in a protection to certain physical and chemical agents. This suggests the possibility of so-called natural resistant forms as distinguished from spore forms. The latter view appears to be favored by the exceptional resistance of tubercle bacilli to drying, extreme vacuums (Potter, 1935 and 1937), and their ability to remain viable for long periods of time under favorable conditions (Corper and Cohn, 1933). Weinzirl (1933), however, found no material protection from heat and chemical agents when the fat content had been increased.

In an earlier paper representing a study of the thermolability of the tubercle bacillus (Corper and Cohn, 1937), we presented an improved method of determining the survival of tubercle bacilli after exposure to heat and noted their high resistance to temperatures affecting other microörganisms detrimentally. At

60°C., fine suspensions (1 mgm.) of human tubercle bacilli in a buffered isotonic solution of pH 7.0 were killed within a few minutes; while at 50°C., the bacilli survived for 5 hours; and at 45°C., for 8 days. The importance of exact quantitative estimations of the suspensions of the bacilli and control of the conditions of experiment were also stressed. Occasionally, and for unexplained reasons, a test would give a survival time differing from that found in the ordinary run of experiments even though young actively growing cultures were used for making the suspensions. In order to gain a further insight into this unusual phenomenon which made some of our data vary in consistency, further observations were planned to learn, if possible, the mechanism of this variation in the cultures of the bacilli.

EXPERIMENTAL PART

The apparatus used in the heat tests was essentially the same as that used in the earlier studies (Corper and Cohn, 1937) with a few additional improvements in refinement. A more delicately controlled heat box was prepared, capable of simultaneously accommodating two test exposures and maintaining the contained fluid exactly at the same temperature as indicated by two thermometers immersed in each bottle and readily read from the outside of the box (see fig. 1). In addition, the pipettes used for removing samples for test were prepared with traps so that the liquid containing the bacilli remained entirely in the exposure bottle (see fig. 2). The technic and method used in the heat tests were essentially the same as that reported in the previous paper (Corper and Cohn, 1937). In order to control the apparatus and prove the consistency of duplicate tests, the same suspension or two separate suspensions made from the same culture tube or two separate suspensions made from different culture tubes of the same age and on the same medium were run at 55°C., side by side in the two test bottles. The results showed no significant variations under these different conditions, e.g., human tubercle bacilli (human avirulent, 3 weeks old, grown on inspissated broth whole egg medium, 1 mgm. per cubic centimeter) from two different culture tubes showed absence of via-

bility only after one-and-a-quarter hours. At the one-hour interval, viability still persisted. Likewise, duplicate suspensions (1 mgm. per cubic centimeter) made from the same culture tube also showed no appreciable difference in susceptibility to heat exposure. Using these preliminary experiments as controls,

FIG. 1

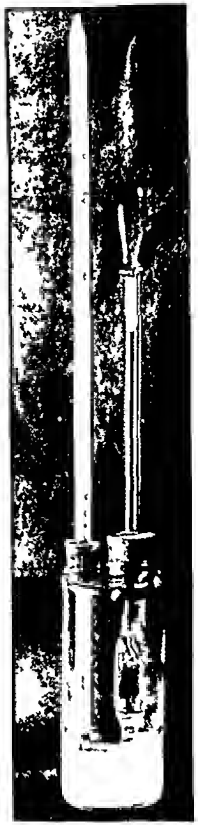

FIG. 2

suspensions made from different mediums were studied for their survival time after exposure to heat and chemicals.

The earliest clue to a varying resistance of human tubercle bacilli grown on different mediums was noted during the course of a study on the thermolability of tubercle bacilli. At that time, all tests were performed with whole egg medium without

broth. Since then, and in later duplication tests, broth was added to some of the mediums with a consequent increase in the survival time of the bacilli. It had been noted previously that the addition of broth to a medium for the cultivation of small numbers of human tubercle bacilli was of no significance (Corper and Cohn, 1933) and it was therefore omitted from the inspissated egg yolk medium recommended for discerning the presence of small numbers of tubercle bacilli. It was recognized, however, that when the weight of bacilli to be obtained from a culture was a factor, the addition of broth resulted in a heavier final growth as compared with the amount obtained in broth-free mediums of like composition. The following results are illustrative of such findings: the moist weight of bacillary growth obtained from a tube of inspissated broth whole-egg medium after 6 weeks was 0.249 gram; while on the broth-free medium, the moist weight was 0.056 gram. In other words, it requires four tubes of inspissated broth-free whole-egg medium to yield as much culture mass as one tube of inspissated broth whole-egg medium.

When determined by dehydration over phosphoric anhydride in a vacuum desiccator, the water content of the bacillary mass was practically identical for the cultures on the broth and the broth-free medium, each averaging about 73 per cent moisture.

THE THERMAL RESISTANCE OF TUBERCLE BACILLI OBTAINED FROM VARIOUS MEDIUMS AND UNDER VARIOUS CONDITIONS

To determine the thermal resistance of human tubercle bacilli (grown with or without broth on whole-egg mediums containing glycerol), several plantings (5 or 6) of the same strains were made on the respective medium. Cultures of the same age and grown under the same conditions were then used for making fine suspensions. The results of the survival time of virulent human tubercle bacilli, strain H-7, after exposure to heat (53° and 55°C.) is recorded in table 1.

Briefly, it is to be noted from table 1 that at 53°C. virulent human tubercle bacilli (H-7) taken from broth containing whole-egg medium survive for $1\frac{1}{2}$ hours, while the bacilli taken from the broth-free whole-egg medium survive for only 45 minutes.

At 55°C., the culture on broth survives for 45 minutes, while on the broth-free culture it survives for only 15 minutes. That this is not a function of the number of viable bacilli in the original suspensions is shown by the control dilution test in which growth occurred in a dilution of one-hundred-millionth of a milligram in all cases.

In a similar experiment, a comparison of thermal resistance was made between the bacilli grown on inspissated broth whole-egg medium and those grown on broth agar medium (standard glycerol agar medium which requires heavy plantings of 1 mgm. per cubic centimeter or more for successful culture). On the agar medium, the weight of culture yield was large compared with other mediums, yet the water content of the bacillary masses showed no appreciable difference: 74.8 and 73.5 per cent respectively for glycerol broth agar and inspissated broth whole-egg medium.

TABLE 1

Thermal resistance of virulent human tubercle bacilli (H-7) cultured on inspissated broth whole-egg medium as compared with the same strain cultured without broth

GLYCEROL WHOLE-EGG MEDIUM	TEMPERATURE†	SURVIVAL TIME								CONTROL DILUTION IN MILLIGRAMS FOR VIABILITY OF BACILLI IN THE SUSPENSIONS		
		Immediately	15 minutes	30 minutes	45 minutes	1 hour	1¼ hours	1½ hours	1¾ hours	10^{-4}	10^{-6}	10^{-8}
	°C.											
With broth.......	53	2*	3	3	3	3	3	4^{2}	0	2	3	4
Without broth....	53	2	3	4	5^{1}	0	0	0	0	2	3	4
With broth.......	55	2	3	3	4^{1}	0	—	—	—	2	3	3^{2}
Without broth....	55	2	4^{2}	0	0	0	—	—	—	2	3	5^{1}

* The numeral given indicates the number of weeks when first appearance of growth was noted (3 culture tubes being used for each test). When used, the exponent indicates the number of tubes of the three which were positive.

† The subcultures used for this experiment were one month old. The concentration of the suspension as tested was 1 mgm. of bacilli per cubic centimeter.

The results of the thermal resistance experiments with these bacilli are recorded in table 2.

A comparison was also made of the thermal resistance (53° and 55°C.) of fine suspensions of avirulent human tubercle bacilli grown on glycerol broth agar and inspissated broth whole-egg medium. At 53°C. the egg medium cultures survived 1¾ hours, and the broth agar medium cultures survived only 30 minutes. At 55°C. the egg medium cultures survived one hour, and those taken from broth agar survived only for 15 minutes. All controls

TABLE 2

Thermal resistance of avirulent human tubercle bacilli cultured on inspissated broth whole-egg medium as compared with the same strain on broth agar medium

MEDIUM	TEMPERATURE†	SURVIVAL TIME									CONTROL DILUTION IN MILLIGRAMS FOR VIABILITY OF BACILLI IN THE SUSPENSIONS		
		Immediately	15 minutes	30 minutes	45 minutes	1 hour	1¼ hours	1½ hours	1¾ hours	2 hours	10^{-4}	10^{-6}	10^{-8}
	°C.												
Broth whole-egg*...	53	2‡	3	3	3	3	4	5^2	5^2	0	2	3	4
Broth agar..........	53	3	4	5^2	0	0	0	0	0	0	3	3	3
Broth whole-egg....	55	2	3	3	4	4^2	0	—	—	—	2	3	3
Broth agar..........	55	2	8^2	0	0	0	0	—	—	—	2	3	3

* All these mediums contained glycerol.

† The subcultures used for the 53°C. test were one month old and for the 55°C. tests, eighteen days old. The concentration of the suspensions as tested was 1 mgm. of bacilli per cubic centimeter.

‡ The numeral given indicates the number of weeks when the first appearance of growth was noted (3 culture tubes being used for each test). The exponent, when used, indicates the number of tubes of the three which were positive.

showed growth in dilutions of the suspensions to one-hundred-millionth milligram per cubic centimeter.

Of the factors which may play a part in the resistance of the tubercle bacilli, the age of the culture is particularly significant. Therefore, the same strain of virulent human tubercle bacilli was planted at different intervals so as to be 3 and 6 weeks old at time of test. These were compared at 53° and 55°C., and some of the results are recorded in table 3.

A comparison of the thermal resistance of the cultures grown

for 3 and 6 weeks showed no appreciable differences at 53° and 55°C. At 53°C., the young culture was slightly more resistant to heat.

In a series of experiments to determine the effect of the age of the suspension as a factor in the resistance of the bacilli to heat, it was found that a suspension allowed to remain at room temperature for one or two days after preparation showed a slight increase in resistance, up to two days, while it became slightly less than that of the original suspension at five days. This phenomena was not a function of agglutination of the suspension

TABLE 3

Thermal resistance of virulent human tubercle bacilli (H-7) cultured for different time intervals on inspissated broth whole-egg medium

AGE OF CULTURE	TEMPERATURE	SURVIVAL TIME										CONTROL DILUTION IN MILLIGRAMS FOR VIABILITY OF BACILLI IN SUSPENSIONS		
		Immediately	15 minutes	30 minutes	45 minutes	1 hour	1¼ hours	1½ hours	1¾ hours	2 hours	2¼ hours	10^{-4}	10^{-6}	10^{-8}
weeks	*°C.*													
3	53	2*	2	2	2	2	3	3	4	7^{2}	0	2	2	3
6	53	2	3	3	3	4	3^{2}	7	0	0	0	2	2	3
3	55	2	3	5^{2}	0	—	—	—	—	—	—	3	3	4
6	55	2	3	4^{2}	0	—	—	—	—	—	—	2	3	4

* The numeral given indicates the number of weeks when the first appearance of growth was noted (3 culture tubes being used for each test). When used, the exponent indicates the number of tubes of the three which were positive.

from standing since the growth limit tests revealed practically the same growth extinction dilution (down to 10^{-8} mgm. per cubic centimeter dilution) for the original (control) and test (time interval) suspension.

THE RESISTANCE OF TUBERCLE BACILLI OBTAINED IN VARIOUS WAYS TO ACETIC ACID

In earlier experiments, it was found that human tubercle bacilli are particularly susceptible to the action of acetic acid (Corper and Uyei, 1930; and Cohn, 1933). For this reason and because

of the favorable time of lethal action of the acetic acid, we chose this agent for testing the resistance of the tubercle bacilli to chemicals.

The methods used are essentially the same used in a previous study (Cohn, 1934). Briefly: to 0.5 cc. of 5 per cent acetic acid was added 0.5 cc. of fine suspension of tubercle bacilli containing 0.1 mgm. of bacilli per cubic centimeter. The mixture was incubated at 37°C. for various time intervals, the action of the

TABLE 4

The effect of 2.5 per cent acetic acid on human tubercle bacilli cultured on inspissated broth whole-egg medium as compared with the same strains cultured on the same medium without broth and on glycerol broth agar

GLYCEROL MEDIUMS	STRAIN* OF BACILLI	SURVIVAL TIME					CONTROL DILUTION IN MILLIGRAMS FOR VIABILITY OF BACILLI IN THE SUSPENSIONS		
		Immediately	30 minutes	45 minutes	1 hour	1½ hours	10^{-4}	10^{-5}	10^{-6}
Broth whole-egg.....	H-7	2†	3	4	5^1	0	2	3	4
Whole-egg...........	H-7	2	5^1	0	0	0	2	3	5
Broth whole-egg.....	Avirulent human	2	3	3	5^2	0	2	3	3
Broth agar...........	Avirulent human	2	4^2	6^1	0	0	3	3	4

* The cultures of H-7 were 25 days old and of "avirulent human" were 5 weeks old.

† *The numeral given indicates the number of weeks when the first appearance of growth was noted* (3 culture tubes being used for each test). When used, the *exponent indicates the number of tubes of the three which were positive.*

acetic acid was interrupted by dilution to 50 cc. by means of 0.9 per cent sodium chloride solution, and finally neutralized with 0.6 cc. 6 per cent sodium hydroxide solution. The final reaction was slightly alkaline to a phenolphthalein indicator. The viability of the bacilli was determined by planting on an imspissated egg yolk medium (Corper and Cohn, 1933).

In table 4 are recorded the results of the lethal action of acetic acid on fine suspensions of avirulent and virulent human tubercle

bacilli obtained from inspissated broth whole-egg medium, the same medium without broth, and glycerol broth agar.

The experiments recorded in table 4 give illustrative results, indicating that bacilli obtained from inspissated whole egg mediums containing broth are more resistant to the lethal action

TABLE 5

The effect of 2.5 per cent acetic acid on virulent human tubercle bacilli cultured for different time intervals on inspissated broth whole-egg mediums

AGE OF CULTURE	SURVIVAL TIME						CONTROL DILUTION IN MILLIGRAMS FOR VIABILITY OF BACILLI IN THE SUSPENSIONS		
	Immediately	30 minutes	45 minutes	1 hour	1¼ hours	1½ hours	10^{-4}	10^{-6}	10^{-8}
weeks									
3	2*	3	4^2	5^1	5^1	0	2	2	3
6	2	3	3	4	4^1	0	2	2	3

* The numeral given indicates the number of weeks when the first appearance of growth was noted (3 culture tubes being used for each test). When used, the exponent indicates the number of tubes of the three which were positive.

TABLE 6

The survival time of human tubercle bacilli as compared with avian tubercle bacilli cultured on broth whole-egg medium

TEMPERATURE OF EXPOSURE		HUMAN* TUBERCLE BACILLI	AVIAN TUBERCLE BACILLI
°C.	*°F.*		
50	122	5 hours	5⅓ + days
55	131	1 hour	8 hours
60	140	15 minutes	30 minutes
65	149	Almost immediately	Within 15 minutes

* The suspensions used in these experiments contained 1 mgm. of bacilli per cubic centimeter.

of acetic acid than the same bacilli cultured on either inspissated whole-egg without broth or glycerol broth agar. It shows the same increased resistance to acetic acid noted previously for heat.

In a manner identical to those on thermal resistance, the experiments on the resistance to the action of acetic acid of cultures of varying ages revealed no significant differences as shown in table 5.

THE THERMAL RESISTANCE OF AVIAN TUBERCLE BACILLI

In an earlier paper, we reported the thermal-resistance of human tubercle bacilli when cultured on inspissated whole-egg medium without broth. At that time no comparison between human and avian tubercle bacilli on the same medium was given. Therefore, it was of interest to compare the thermal resistance of a strain of newly isolated avian tubercle bacilli (isolated from the liver of a chicken in 1936) with a strain of human tubercle bacilli on the same medium (inspissated broth whole-egg). The results of such tests are briefly recorded in table 6.

A comparison of the thermal resistance of fine suspensions of avian and human tubercle bacilli shows the avian strain to be decidedly more resistant to the lethal effects of heat.

SUMMARY AND CONCLUSIONS

In studying the thermal and chemical (acetic acid) resistance of human tubercle bacilli, it was found that the type of medium on which the bacilli had been cultured was a determining factor in the resistance of these bacilli. Those cultured on egg mediums containing broth usually showed a greater resistance to heat and acetic acid than those cultured in the absence of broth. Likewise, bacilli cultured on whole-egg mediums with broth showed a greater resistance than those cultured on glycerol broth agar. The age of the culture, within certain limits of from 3 to 6 weeks, appeared to exert no appreciable effect on the resistance of the bacilli. There was no apparent relation between the ability of a medium to support the growth of small numbers of tubercle bacilli and thermal lability or resistance to acetic acid. The use of varying types of mediums, besides errors in technic, probably account for the wide variations in thermal resistance noted for tubercle bacilli in the literature. The thermal survival time of avian tubercle bacilli exceeded that noted for human tubercle bacilli under the same conditions of culture and heat exposure.[1]

[1] We are grateful to Mr. L. D. Miller for assisting with the technical phases of this study.

REFERENCES

COHN, M. L. 1933 An analysis of Loewenstein's method for isolating tubercle bacilli from blood. Jour. Infect. Dis., 52, 214–222.

COHN, M. L. 1934 The antiseptic effect upon tubercle bacilli of certain advocated compounds. Jour. Bacteriol., 27, 517–526.

CORPER, H. J., AND UYEI, NAO 1930 Oxalic acid as a reagent for isolating tubercle bacilli and a study of the growth of acid-fast non-pathogens on different mediums with their reactions to chemical reagents. Jour. Lab. and Clin. Med., 15, 348–369.

CORPER, H. J., AND COHN, M. L. 1933 The nutrient quality of eggs for growing tubercle bacilli. Amer. Jour. Hyg., 18, 1–25.

CORPER, H. J., AND COHN, M. L. 1933 The viability and virulence of old cultures of tubercle bacilli. Amer. Rev. Tuberc., 28, 857–874.

CORPER, H. J., AND COHN, M. L. 1937 The thermolability of the tubercle bacillus. Amer. Rev. Tuberc., 35, 663–677.

POTTER, T. S. 1935 The survival of tubercle bacilli subjected to a vacuum of high order. Jour. Infect. Dis., 57, 149–159.

POTTER, T. S. 1937 Survival of oxygen and water deprival by tubercle bacilli. Jour. Infect. Dis., 60, 88–93.

WEINZIRL, J. 1933 Biology of the tubercle bacillus. III. Does the so-called fatty capsule serve to protect the tubercle bacillus. Jour. Bacteriol., 25, 447–459.

STUDIES ON CULTURAL CHARACTERISTICS, PHYSIOLOGY AND PATHOGENICITY OF STRAIN TYPES OF PHYTOMONAS STEWARTI[1]

S. S. IVANOFF, A. J. RIKER AND H. A. DETTWILER

The Department of Plant Pathology, University of Wisconsin

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I. INTRODUCTION

Interest in bacterial wilt or Stewart's wilt disease of corn (*Zea mays* L.) caused by *Phytomonas stewarti* (Smith) Bergey et al. has recently been stimulated by several epidemics, especially that of 1932, which caused serious injury to field corn and the destruction of entire fields of Golden Bantam sweet corn in many parts of the United States. This epidemic was remarkable because of its severity and extent and because of certain symptoms found. Besides the now well known diagnostic symptom of leaf striping which, until that time, was not emphasized and hardly mentioned by the earlier workers (Stewart, 1897, Smith, 1914), other symptoms were evident, such as dry cavities or wet rotting in the stalk, blackening of the lower portion of young plants, and other less conspicuous symptoms, which caused some doubt as to whether this was the true Stewart's disease described earlier. In addition the cultures from diseased tissue showed some characteristics unlike those described by Smith (1898, 1901, 1914). These and related problems have stimulated studies by a number of investigators (Ivanoff, 1933a, 1933b, 1935, 1936; Ivanoff and Riker, 1936; Wellhausen, 1935; Elliott, 1935; Frutchey, 1936, and others).

The existence of bacterial wilt organisms differing in degree of pathogenicity and in some cultural and physiological characters

[1] Approved for publication by the Director of the Wisconsin Agricultural Experiment Station. The photographs were made by Eugene Herrling.

was reported by Ivanoff (1933a, 1933b). Holbert et al. (1933) noted strains causing "bacterial leaf blight of dent corn," apparently the striped leaf condition induced by *P. stewarti*. McCullock (1918) noted two types of yellow colonies from corn infected with Stewart's disease. One had a smooth, flat surface, the other a definite central depression, resembling a crater. However, the organisms appeared identical in virulence, character of infection, general cultural characteristics, and morphology, including the lack of flagella.

The present studies deal mainly with: (1) morphologic and staining characteristics, (2) cultural characteristics on various media, (3) physiological and physico-chemical characteristics, (4) degrees of pathogenicity, (5) correlation between amount of gum produced and pathogenicity, and (6) occurrence and distribution of the strains in the United States.

II. SOURCE AND ORIGIN OF THE CULTURES

The cultures were isolated from many varieties of corn in various localities at different times. Nineteen cultures were progenies of single cells, and three, each representing a different strain type, were derived from colonies and were included for comparison. The cultures were tested for pathogenicity on inbreds and crosses from the Golden Bantam variety. Most of the determinations were made with all the cultures, but a few were conducted with representative type cultures as mentioned in the text. A list of the cultures with their histories is given in table 1. The 22 cultures have been grouped, tentatively, on the basis of their several characteristics into types A, B, and C.

III. MORPHOLOGY OF THE BACTERIAL CELLS AND THEIR STAINING REACTIONS

The organisms were all small rods with rounded ends. Measurements on negative nigrosin preparations were made with a filar micrometer on 100 cells of 48-hour-old and 4- to 5-week-old cultures, respectively, grown on nutrient glucose agar slants at 24°C. No significant differences were found among the various 48-hour-old cultures in the average size of the individual cells,

the dimensions varying from 0.4 to 0.8μ in width and from 0.9 to 2.2μ in length, with an average of 0.6 by 1.6μ. Conspicuous differences were noted after 4 to 5 weeks, however, in the length of the cells in three groups of cultures (characterized later). The cells of type C ranged from about 0.8 to 5.0μ in length,

TABLE 1

Origin of Phytomonas stewarti cultures used

TYPE	DESIGNATION OF CULTURE	CORN FROM WHICH CULTURE WAS ISOLATED	STATE WHERE CORN WAS GROWN	YEAR OF ISOLATION	ORIGIN
A	1	Golden Bantam	Virginia	1931	Single cell
	2	Golden Bantam	Virginia	1931	Colony
	3	Golden Early Market	Wisconsin*	1932	Single cell
	4	Golden Early Market	Wisconsin*†	1932	Single cell
	5	Golden Bantam	Wisconsin	1933	Single cell
	6	Dent corn (inbred)‡	Wisconsin	1933	Single cell
	7	Leaming	Illinois	1933	Single cell§
	8–12	Leaming	Illinois	1933	Single cell¶
B	13	Leaming	Illinois	1933	Single cell§
	14–15	Leaming	Illinois	1933	Single cell‖
	16	Golden Bantam	Illinois	1933	Single cell
	17	Country Gentleman	Illinois	1933	Colony
	18	Dent corn (inbred)‡	Illinois	1933	Single cell
	19	Dent corn‡	Ohio	1932	Single cell
C	20	Dent corn‡	Iowa	1932	Single cell
	21	Golden Glow	Wisconsin	1933	Colony
	22	Golden Bantam	Illinois	1933	Single cell

* Seed from which the plants were grown was obtained from the eastern United States.

† Culture isolated from a larva of *Diabrotica longicornis* Say.

‡ Variety undetermined.

§ Single cell culture derived from a single cell parent culture.

¶ Sister cultures of 7.

‖ Sister cultures of 13.

about 10 to 25 per cent being longer than 3.0μ. The unusually long cells were often beaded. Long cells were noted less frequently in type B than in type C. No long cells were observed in the type A cultures.

Of the strains grown in nutrient broth or on nutrient-glucose-

agar slants none was found to be motile in hanging drops. All were gram-negative and not acid-fast at various stages of growth. No endospores were found.

IV. GROWTH AND COLONY CHARACTERS

Distinguishing characters were noted when the cultures were grown on nutrient glucose agar (composition: beef extract, 3 grams; peptone, 10 grams; glucose, 10 grams; agar, 17 grams;

TABLE 2

Grouping of 22 bacterial wilt cultures into three main strain types on the basis of characters shown on nutrient glucose agar, after 14 days incubation at 24°C.

CHARACTERISTICS	TYPE A (INCLUDING CULTURES 1 TO 12)	TYPE B (INCLUDING CULTURES 13 TO 19)	TYPE C (INCLUDING CULTURES 20 TO 22)
I. On agar plates			
Color	Yellow (orange)	Yellow (lemon)	Yellow (cream)
Diameter of colonies	10 to 12 mm.	8 to 10 mm.	3 to 5 mm.
Consistency	Butyrous to butyrous-viscid	Viscid	Somewhat membranous
Surface	Smooth or crateriform and slightly rough	Smooth or concentrically ringed	Smooth
Elevation of growth	Convex	Raised	Flat
II. On agar slants			
Amount of growth	Abundant	Abundant	Slight
Form of growth	Spreading	Mucoid*	Filiform

* Growth runs down the slant to the bottom of culture tube. For other terms see Society of American Bacteriologists, Committee on Bacteriological Technic. 1933. Pure culture study of bacteria, 1, 10–12.

and water to make up a liter, with pH adjusted to about 7.0), and on other media.

The colony characters varied quantitatively within a type. For example, cultures 6, 7, 8, 9, 10, 11, and 12 of type A had a less intense orange-yellow color and were less viscid than cultures 1, 2, 3, 4, and 5 of the same type.

The growths on nutrient glucose agar slants were also characteristic in amount, form, and consistency (table 2).

The growth of the various cultures in Ivanoff's liquid medium (for composition of medium cf. Ivanoff, 1933b) differed considerably from that on nutrient glucose agar. The cultures of types A and B, and some of type C grew very well in both. Three cultures of type B (13, 14, and 15, all sister single-cell cultures originating from a common mother culture also of single-cell origin), however, made very scanty growth on the solid medium and no growth in the liquid medium.

The data obtained with ammonium-phosphate mineral-salts glucose agar, potato-glucose agar, corn agar, soil agar, prune

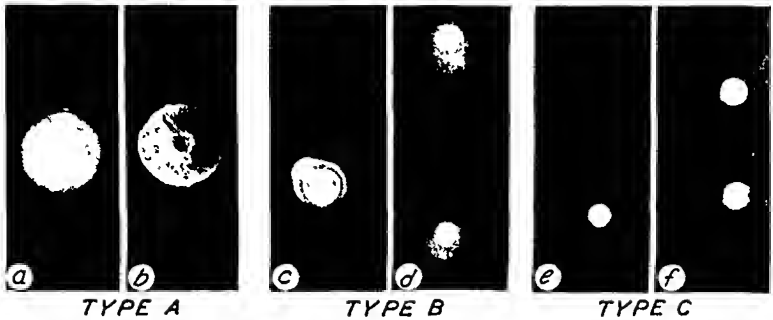

Fig. 1. Colonies of Three Types of Phytomonas stewarti Grown on Nutrient Glucose Agar Plates at 24°C.

The cultures differ in various characters including pathogenicity (see tables 2, 4 and 5).

agar, and cabbage-extract agar have no additional determinative significance and are omitted.

Other tests were made as follows:

Gelatin stabs (10 per cent gelatin in water, at pH 7.0, and incubated at 20°C.) were made with all cultures. The growth after 3 weeks' incubation was slight but uniform, the line of puncture was filiform, and no liquefaction was observed. The medium appeared unchanged.

The litmus milk test (90 grams of powdered skim milk plus litmus, added to a liter of water) showed no great differences among the various cultures after three weeks' growth at 28°C. The pH varied with the cultures from about 5.5 to 6.5, with slight

changes of color induced in the medium. No curd or serum zone was noted.

Production of indol was tested with Bohme's solution (medium used: beef extract, 3 grams; tryptophane peptone (Difco), 10 grams; and water to make a liter, with pH adjusted to 7.0). *Indol was absent from all cultures except* those used as controls on this test.

None of the cultures hydrolyzed starch in Petri dish cultures.

These four tests failed to demonstrate any significant differences among the cultures studied, but are useful in defining characteristics apparently common to the species *P. stewarti.*

V. PHYSIOLOGICAL AND PHYSICO-CHEMICAL TESTS

The ranges of temperatures at which the various wilt cultures grow, as well as the optimum temperatures, were determined. Each culture was run in triplicate in broth and the trials were performed twice. No growth was observed at 4°C., there was only slight growth at 8° and 12°C., and only a few cultures showed perceptible growth at 37°C. The optimum temperature for the various cultures ranged from 28° to 32°C. The slight differences noted among the various cultures were not considered significant.

The optimum pH was near 7.0 Abundant growth of all cultures in nutrient glucose broth was observed after two weeks from pH 6.0 to pH 8.0. On the acid side growth decreased at 5.5 but was still marked at 4.5 and absent at 4.0 from all cultures. On the alkaline side growth decreased at about 8.5.

Fermentation of various carbon sources by the 22 wilt cultures was investigated with three basic media, nutrient broth, ammonium-phosphate mineral-salts medium, and yeast-water mineral-salts medium.[2] The carbon sources tested are listed in table 3.

[2] The composition of these media was as follows:

Nutrient broth—Parke, Davis and Co peptone, 10 grams; Armour's beef extract, 3 grams; carbon source, 10 grams; and water to make a liter

Ammonium-phosphate-mineral-salts medium—ammonium phosphate (*monobasic*), 10 grams; potassium chloride, 0 2 gram; magnesium sulphate, 0 2 gram; carbon source, 10 grams; and water to make a liter.

Yeast-water-mineral-salts medium—yeast water extract, 100 cc ; magnesium sulphate, 0.2 gram; sodium chloride, 0 2 gram; potassium phosphate (dibasic), 0.2 gram; calcium chloride, 0 1 gram; carbon source, 10 grams, and water to make a liter.

The basic media and the carbon sources were adjusted to pH 7.0, sterilized separately, then mixed, and the reaction again checked and readjusted if needed. Each source of carbon was tested in each of the basic media in triplicate with the various cultures from 2 to 5 times. The pH readings were taken with a

TABLE 3

*Hydrogen-ion concentration induced after 3 weeks at 24°C. by Phytomonas stewarti cultures in nutrient broth with stated sources of carbon**

TYPE	CULTURE NUMBER	CARBON SOURCE													NO ADDED CARBON SOURCE
		Glucose	Levulose	Galactose	Lactose	Sucrose	Maltose	Starch	Inulin	Dextrin	Glycerol	Mannitol	Salicin	Pectin	
A	1	6.0	6.0	6.0	5.9	6.3	8.2	7.9	7.8	8.4	6.6	7.0	7.6	7.7	7.9
A	2	6.0	6.1	6.5	5.8	6.0	7.9	8.4		8.2	6.8	6.0			8.4
A	3	5.8	5.6	5.9	5.8	5.9	8.6	8.2		8.3	6.9	6.7	7.7	7.7	8.3
A	4	5.8	6.0	5.9	5.8	6.0	8.5	8.4		8.5	6.9	6.1			8.6
A	5	5.5	5.7	5.7	5.7	5.7	8.8	8.6		8.5	6.9	5.8			8.9
A	6	5.4	5.6	5.8	5.7	5.3	9.0	8.8		8.2	6.6	6.9	7.7	7.6	8.3
A	7	5.4	5.5	6.0	6.3	6.9	8.2	8.2	7.8	7.7	6.6	6.9	7.8	7.7	8.2
A	10	5.4	5.6	6.4	5.9	6.9	8.2	7.3	7.8	8.3	6.6	6.0	7.2	7.8	8.3
B	14	5.5	5.2	5.7	5.9	6.9	8.3	8.2	7.8	8.3	6.4	6.5	7.8	7.8	8.3
B	16	5.4	5.3	5.5	5.1	5.6	8.5	7.2		8.2	6.4	6.7	7.5	7.7	8.4
B	17	5.2	5.2	5.5	6.0	5.4	8.2	7.7	7.8	8.4	6.9	6.9	7.7	7.7	8.2
B	18	5.3	5.1	5.8	4.8	5.6	8.6	8.2		8.4	6.8	7.1	7.7	7.8	8.3
B	19	5.4	5.6	6.0	6.0	5.7	8.6	8.7		8.8	6.7	5.6			8.6
C	20	5.6	5.7	6.5	5.8	5.4	8.3	7.7		8.0	6.2	6.1	7.6	7.7	8.1
C	21	4.9	5.3	5.8	5.7	5.4	7.4	7.1	7.7	7.4	6.5	6.0	7.3	7.7	7.5
C	22	5.4	5.3	6.1	6.0	5.2	8.2	8.2		8.2	6.3	6.8	7.4	7.7	
None		7.0	6.9	6.9	7.0	7.0	7.0	7.0		7.0	7.1	7.1		7.1	7.0

* The composition of this medium is given in the text. Determinations on several sister single-cell cultures are omitted.

quinhydrone electrode usually after 3 weeks of incubation, although in some trials readings were also taken after one and four weeks. A summary of results of trials with nutrient broth, which serves as a representation of the other studies, is given in table 3. The data in this table not only show the variations among the cultures but also define the reaction of the species as a

whole. These studies on carbon sources may be summarized as follows: (1) Acid reaction was induced by all cultures in media containing glucose, levulose, galactose, lactose, and glycerol. (2) Alkaline reaction or no change was induced by all cultures in media containing maltose, starch, inulin, dextrin, salicin, and pectin, and in the basal media containing no added source of carbon. (3) With sucrose and mannitol all the cultures were acid after one week. However, the reaction varied after 3 weeks as discussed later.

The amount of growth in these media differed with the source of carbon and the culture. As a rule all cultures of types A and B grew better with all carbon sources than the cultures of type C. Likewise, the cultures grew very well with glucose, levulose, galactose, lactose, sucrose, glycerol and mannitol but produced moderate to slight or no growth with maltose, starch, inulin, dextrin, salicin, pectin, and in the basal media containing no added carbon source.

Quantitative determinations of glucose utilized by the various cultures were also made. The method employed was that of Shaffer-Hartmann as modified by Stiles, Peterson, and Fred (1926). All cultures were grown in nutrient broth with 1 per cent glucose. Three trial cultures of each, in triplicate, were made. At the end of a 3 weeks' incubation period, all cultures had used approximately 50 per cent of the sugar. There was a tendency for the cultures of type A to use slightly more sugar within that incubation period than those of the types B and C.

The length of the incubation period in nutrient glucose broth had considerable effect on the hydrogen-ion concentration induced as shown in figure 2, which is prepared from the averages of three trials with all cultures. In these trials the cultures were more active than those reported in table 3, probably owing to better aeration. Doubtless the same shift toward a more alkaline reaction would occur in those other cultures with longer incubation. Type A cultures were sharply differentiated after four weeks from types B and C cultures, the last two acting similarly. Almost identical results were secured in like studies with carrot extract (200 grams carrots steamed in 1 liter of water).

In nutrient broth containing no glucose, all of the cultures produced at the end of the first week a pH of about 7.4 which rose to about 7.8, 8.0, and 8.2 at each of the three subsequent weekly intervals.

The similarities between the cultures of *P. stewarti* and *Phytomonas vascularum* (Cobb) Bergey et al., the causal organism of

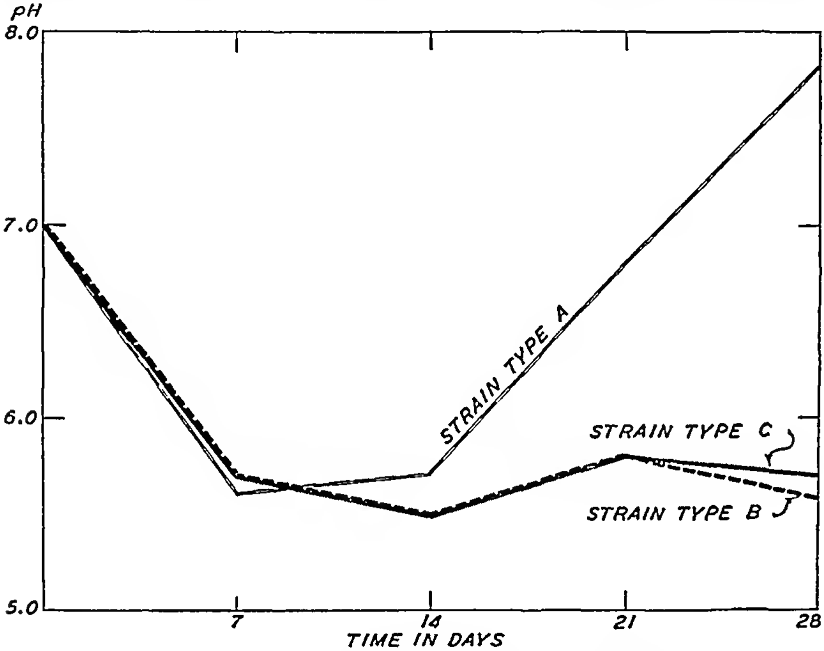

FIG. 2. CHANGES IN THE HYDROGEN-ION CONCENTRATION OF NUTRIENT GLUCOSE BROTH, INDUCED AT SUCCESSIVE INTERVALS BY THE GROWTH OF DIFFERENT STRAIN TYPES OF PHYTOMONAS STEWARTI

The reaction, pH 7.0, of the unseeded control tubes remained unchanged.

Cobb's disease of sugar cane (*Saccharum officinarum* L.), may deserve a brief mention. Ivanoff (1935) reported that the sugar cane organism can induce symptoms on corn and sorghum similar to those induced by the various cultures of *P. stewarti*. These similarities in pathogenicity between the two bacterial species prompted the writers to include a culture of *P. vascularum* in some of the cultural and physiological tests. However, be-

cause of the limited trials and because of the use of only one culture of the sugar cane organism a comparison between the two species is omitted. As far as the studies went it appeared that this culture was similar in behavior to some cultures of the B and C types of *P. stewarti*. The question of similarities between *P. stewarti* and a number of other yellow vascular parasites on grasses offers a promising field of investigation.

VI. DIFFERENCES IN PATHOGENICITY AMONG THE STRAINS

Degrees of pathogenicity characteristic of the cultures were observed in the greenhouse with all the cultures studied, on inoculating by puncture young corn plants of the Golden Bantam and Leaming varieties. All cultures tentatively placed in types A and B induced more or less severe infection, many of the plants dying in less than 3 weeks after inoculation, while cultures belonging to type C induced noticeably less severe infection. The tests were continued in the field during 3 seasons. One to several representatives of each type were inoculated into five standard kinds of sweet corn, Golden Gem, Golden Bantam, Purdue 1339 (an inbred), Golden Cross (a hybrid), and Country Gentleman, with susceptibility decreasing in the order named (Ivanoff (1936)). The inoculations and the estimate of degrees of pathogenicity were performed under conditions and with precautions previously adopted by Ivanoff and Riker (1936) in their wilt disease resistance studies. All cultures of type A were uniformly highly pathogenic, causing a reduction of approximately 75 per cent in yield of the Golden Bantam corn. The cultures of type C were uniformly low in pathogenicity, the disease never causing more than 25 per cent reduction in yield on Golden Bantam corn, often considerably less. The cultures of type B, however, seemed to differ among themselves in regard to pathogenicity; some were comparable to cultures of type A, some were even more pathogenic, while others were distinctly less pathogenic, causing a reduction in yield of Golden Bantam corn ranging from about 50 to 75 per cent. One freshly isolated culture of the B type, not included in these studies, caused the death of 3 foot plants of the Golden Glow variety in less than a week after inoculation. Representative results of these tests are given in

table 4. The relationship between the degrees of pathogenicity of various strains of this pathogen has been more or less maintained with all the corn varieties tested. These strains, unlike

TABLE 4

Degree of pathogenicity of bacterial wilt cultures representing three main strain types of Phytomonas stewarti tested during 1933, 1934, and 1935 on five kinds of corn of varying susceptibility

BACTERIAL CULTURE TESTED	HOST INOCULATED	INDEX OF PATHOGENICITY*		
		1933 trials	1934 trials	1935 trials
1 (Type A)	Golden Gem	100	100	100
	Golden Bantam	72	81	78
	Purdue 1339	63	75	70
	Golden Cross	51	63	59
	Country Gentleman		21	24
14 (Type B)	Golden Gem	100	95	90
	Golden Bantam	64	72	73
	Purdue 1339	59	64	65
	Golden Cross	43	61	50
	Country Gentleman		29	10
22 (Type C)	Golden Gem	40	32	33
	Golden Bantam	16	23	21
	Purdue 1339	15	16	18
	Golden Cross	10	10	8
	Country Gentleman	0	0	0

* The index of pathogenicity was estimated on the basis of injury resulting from the disease to 200 plants for each corn variety at the time of harvesting for canning. Each inoculated plant was given one of the following five indices of susceptibility: 0, 25, 50, 75, and 100, corresponding to relatively distinct degrees of disease injury. The index of pathogenicity for a culture was stated as the mean of the indices of all the plants of that variety. It represented roughly the per cent reduction in yield on account of the disease. For detailed description of these indices see Ivanoff and Riker (1936).

some of the physiological forms of *Puccinia graminis* Pers., have not shown host specialization.

VII. KINDS OF SYMPTOMS INDUCED BY THE VARIOUS STRAIN TYPES

The characteristic symptoms induced by the three types of *Phytomonas stewarti* were studied during different seasons under a

variety of conditions, by inoculations on several thousand corn, sorghum (*Holcus sorghum* L.), and Sudan Grass (*Holcus sudanensis* Bailey) plants of different varieties. The results of these trials may be summarized as follows: (1) All types produced on the same host the same type of leaf symptoms described earlier in detail by Ivanoff (1935). (2) So far, no blackening of the lower part of the stem of young plants, or wet rotting (See Ivanoff, 1935, fig. 3) of the culm tissue has been induced under controlled conditions in the greenhouse or in the field by type A. (3) Dry cavities in the culm of several corn varieties tried were observed in different seasons on about 1 per cent of the corn plants inoculated with cultures of types A and B. (4) Blackening of the lower parts of young plants and wet rotting of the culms have been observed in some seasons in approximately 1 to 20 per cent of the plants inoculated with cultures of type B, but in most of these cases another bacterial organism, apparently non-pathogenic when alone, has been found associated with the wilt bacteria, as already reported (Ivanoff (1935)).[3] (5) Small pockets in the pith tissue of the corn stalk filled with a pure culture of the bacteria have been frequently found more or less associated with all culture types. The above results indicate that, while there is a tendency for the B type of cultures, under certain conditions not yet well understood, to induce symptoms in the corn stalk tissue different from those induced by the other culture types, the evidence is inadequate at present for differentiating the various culture types.

VIII. CORRELATION OF DEGREE OF PATHOGENICITY AND AMOUNT OF GUM PRODUCED BY VARIOUS CULTURES

The pathogenicity of cultures of the B and C types appeared associated in general with the amount of gum produced on nutrient agar containing glycerol and on other media, both solid and liquid. This relation was tested with groups of cultures selected for the different relative amounts of gum produced in various media. In these trials the relative amount of gum pro-

[3] Frutchey (1936) reported a white bacterial organism pathogenic on corn, frequently found associated with *P. stewarti*.

duced was estimated by the flow of the test tube contents, consisting of medium, bacteria, and their products. The results of inoculations made with these cultures on Golden Bantam corn in the field are shown in table 5. These results indicate a promising field for further investigation.

IX. VARIABILITY OF INDIVIDUAL CULTURES AND STRAIN TYPES

The degree of variability within each type was examined. The cultures of type A remained unchanged in colony characters through a period of at least five years with one exception, al-

TABLE 5

Relation between amount of gum produced by various cultures of Phytomonas stewarti in nutrient glycerol broth and their degree of pathogenicity for corn

CULTURES*	AMOUNT OF GUM OBSERVED IN CULTURE TUBES	INDEX OF PATHOGENICITY FOR GOLDEN BANTAM CORN†
16	Abundant	93
17	Abundant	96
18	Moderate	84
19	Moderate	75
13	Slight	45
15	Slight	49
20	None	23
21	None	24

* Cultures 20 and 21 belong to type C; the others to type B.

† See footnote under table 4.

ready mentioned. Occasionally culture 2 (not a single-cell culture) produced a number of crateriform colonies of the form shown in figure 1, B. So far no other culture has produced such colonies. Frequent transferring on routine culture media and continuous cultivation induced no variations except a slight reduction in pathogenicity. Within type A some variations were observed. For example, cultures 6, 7, 8, 9, 10, and 12 were slower than other type A cultures in changing the reaction of sugar media. The cultures of type B, except for reduced pathogenicity, also remained unaltered during the first year after their

isolation. Recently, however, some, whether of single cell origin or otherwise, showed variations in the amount of gum produced, and a few of them apparently have changed to type C both in colony character and in pathogenicity. No one of

TABLE 6

The occurrence and geographic distribution of types of Phytomonas stewarti on several varieties of corn from 1932 to 1936

	ORIGIN OF CORN	VARIETY FROM WHICH BACTERIAL CULTURES WERE ISOLATED	TOTAL NUMBER OF CULTURES ISOLATED	STRAIN TYPES ISOLATED		
				A	B	C
				no.	*no.*	*no.*
1932	Virginia	Golden Bantam	14	12	2	None
	Illinois	Golden Bantam	20	4	11	5
	Illinois	Country Gentleman	17	None	16	1
	Illinois	Leaming	28	None	22	6
	Wisconsin	Golden Bantam	86	None	86	None
	Wisconsin	Golden Early Market	25	3*	22	None
	Wisconsin	Golden Glow	100	None	100	None
1933	Illinois	Golden Bantam	119	5	107	7
	Illinois	Golden Cross	17	None	16	1
	Illinois	Golden Gem	12	None	12	None
	Wisconsin	Golden Bantam	22	1	21	None
	Wisconsin	Golden Bantam	16	1	15	None
1934	New Jersey	Golden Bantam	12	6	6	None
1935	New Jersey	Golden Bantam	11	2	9	None
1936	New Jersey	Golden Bantam	10	1	9	None
Total			509	35	454	20

* Evidence suggested that infection of at least one of the three plants from which these cultures were isolated took place from diseased seed brought from an eastern state. Larvae of *Diabrotica longicornis* Say probably introduced the bacteria to the other plants.

these changed cultures has thus far reverted to its original type. The cultures of the C type showed no apparent variations except that when many colonies were plated, occasionally a large, bright yellow colony appeared among the others. Growth of similar appearance has also been observed on agar slants. Transfers

and dilutions made from these unusual colonies, however, produced growth characteristic of the original C type of colonies. Occasional passage through the host plant did not change the colony characteristics of the cultures at any time.

X. OCCURRENCE AND GEOGRAPHIC DISTRIBUTION OF THE BACTERIAL STRAINS

The occurrence and distribution of the three strain types of the bacterial wilt organism on several varieties of corn during five seasons, from 1932 to 1936 was studied. Isolations were made from diseased material collected from localities where the disease was particularly prevalent, and a record was kept of the kind of bacterial wilt cultures found in each locality. These records, summarized in table 6, indicate that (1) certain culture types are more prevalent in one locality than in another in a particular season, (2) type A cultures were at first isolated only from material grown in the Eastern United States, but later were found sparingly in the Middle West, and (3) type C cultures were more rare and were found only in localities where type B cultures were most prevalent. These findings should be considered only as indicative of the occurrence and distribution of the various types of *Phytomonas stewarti* during the particular seasons of observation.

XI. DISCUSSION

It appears from the study of 22 wilt-producing cultures of *Phytomonas stewarti* that this bacterial species includes several types of organisms. The writers consider that for the present (1) no taxonomic sub-divisions should be made in the species, and (2) under the name of *Phytomonas stewarti* should be included a number of non-motile, yellow bacterial organisms, producing stripes and wilting on a number of grass plants, particularly on corn. The three main strain types have been separated on the basis of certain morphological, physiological, and colony characteristics. While further divisions are possible, they are not now considered to serve a useful purpose.

Perhaps the most conspicuous differences between these strain

types are the color, shape, consistency, and size of the colonies on nutrient-glucose-agar plates (fig. 1). These differences are so distinct that the writers have seldom had difficulties with apparently intermediate strain types. While doubtless true intermediates may exist, it is more likely that cultures appearing intermediate are only mixtures of two or three strain types. All colonies of type A (fig. 1, *a* and *b*) appear set apart as representing a distinct type with hardly any qualification except that the kind of colony represented at *b*, produced by a culture not of a single-cell origin, differs from the leading representative colony at *a* in having a slightly rough surface and a crater-like depression in the center. In all other respects noted, these two types of colonies behaved very much alike. The case of the colonies of type B and C is less clear than that of type A, mainly because of the apparent tendency of some cultures of type B to change to type C after cultivation on nutrient glucose agar for some time, and because of the similarities in physiological characteristics studied thus far (fig. 2). For these two reasons, one might consider type B and C cultures as representing a single though unstable type, quite distinct from type A. However, because (1) these two types have been found so frequently in nature, the former more frequently than the latter, (2) many of the cultures have remained stable after cultivation and after occasional passage through the host, and (3) there exist conspicuous differences between them in colony character and degree of pathogenicity, the writers at this time consider the two forms as distinct.

It has been difficult, because of varying methods employed, to determine which strain type or types were prevalent at the time of the discovery of Stewart's disease, and during its investigation by Stewart (1897) and Smith (1898, 1901, 1914). The indications are that these early investigators worked mainly with A type cultures. Likewise the geographic distribution of the various strain types as reported in table 6 suggests that during the later epidemics at least the A type organisms were frequently found in the Eastern States and rarely encountered in the Middle West, while the B type cultures were predominant in the latter

region. There are indications also that the distribution of the strain types changes from season to season, possibly because of introduction of diseased seed to new localities, dissemination by insects, influence of environmental conditions, and other causes.

It appears that, in addition to the influence of environmental factors, the more frequent local occurrence of highly pathogenic forms than of less pathogenic forms should be considered in relation to epidemic outbreaks.

Basic questions concerning pathogenicity may be approached advantageously with Stewart's wilt bacteria. A clarification of the different types of cultures present in nature, and of the importance of single-cell cultures where variations are concerned, seems to be a necessary prerequisite for such investigations.

XII. SUMMARY

A number of single-cell cultures of *Phytomonas stewarti* inducing striping and wilting on corn (*Zea mays* L.), and other grasses have been studied, in regard to a number of morphological and physiological characters including pathogenicity.

Conspicuous differences have been noted among the cultures which permitted their arrangement into three strain types as follows:

Type A. Colonies on nutrient-glucose-agar plates at 24°C. for two weeks; orange-yellow, of butyrous consistency, surface smooth (or occasionally slightly rough with a conspicuous central depression), elevation convex, approximate diameter 10 to 12 mm. All cultures grew very well in Ivanoff's selective medium, and induced in nutrient-glucose broth an acid reaction after 1 week and an alkaline reaction after four weeks' growth. Cultures highly pathogenic and relatively stable.

Type B. Colonies on nutrient-glucose-agar plates at 24°C. for two weeks; lemon-yellow, of viscid consistency, may contain conspicuous amount of gum, surface smooth, rarely concentrically ringed, elevation raised, approximate size 8 to 10 mm. Some cultures of this type grew very well in Ivanoff's medium, others less so, while still others made hardly any growth at all. All cultures induced an acid reaction in nutrient-glucose broth after

both one and four weeks' growth. The cultures varied in their pathogenicity and some changed their colony characteristics to type C after prolonged cultivation on artificial media.

Type C. Colonies on nutrient-glucose-agar plates at 24°C. for two weeks; cream-yellow, consistency somewhat membranous, surface smooth, elevation flat, size 3 to 5 mm. All cultures grew very well in Ivanoff's medium, and produced an acid reaction in nutrient-glucose broth after both one and four weeks' growth. Cultures only slightly pathogenic and relatively stable in their characteristics.

All cultures of each type failed to (1) liquefy gelatin, (2) produce much change in color of litmus milk, (3) produce indol, or (4) hydrolize starch.

Acid reaction was induced by all cultures of each type after 1 week's growth at 24°C. with glucose, sucrose, levulose, galactose, lactose, glycerol, and mannitol, and no change or an alkaline reaction with maltose, starch, inulin, dextrin, salicin, pectin, and basic media without added source of carbon.

Only slight differences in the amount of glucose utilized by the various cultures were found after 3 weeks' growth in nutrient broth.

Certain culture types seemed to predominate during given seasons in certain localities.

A direct relation was indicated between the amount of gum produced by certain cultures and their pathogenicity.

No changes are suggested in the taxonomic status of *Phytomonas stewarti* on the basis of differences found among the various cultures.

REFERENCES

ELLIOTT, C. 1930 Manual of Bacterial Plant Pathogens. Williams & Wilkins, Baltimore, Md. 349 pp.

ELLIOTT, C. 1935 Dissemination of bacterial wilt of corn. Iowa State Col. Jour. Sci., 9, 247-266.

FRUTCHEY, C. W. 1936 A study of Stewart's disease of sweet corn caused by *Phytomonas stewarti*. Mich. Agr. Exp. Sta. Tech. Bul. 152.

HOLBERT, J. R., ELLIOTT, C., AND KOEHLER, B. 1933 Bacterial leaf blight of dent corn. (Abstract.) Phytopath., 23, 15-16.

Ivanoff, S. S. 1933a Bacterial wilt of corn. (Abstract.) Phytopath., 23, 18.

Ivanoff, S. S. 1933b Stewart's wilt disease of corn, with emphasis on the life history of *Phytomonas stewarti* in relation to pathogenesis. Jour. Agr. Research, 47, 749–770.

Ivanoff, S. S. 1935 Studies on the host range of *Phytomonas stewarti* and *P. vascularum*. Phytopath., 25, 992–1002.

Ivanoff, S. S. 1936 Resistance to bacterial wilt of open-pollinated varieties of sweet, dent, and flint corn. Jour. Agr. Research, 53, 917–926.

Ivanoff, S. S., and Riker, A. J. 1936 Resistance to bacterial wilt of inbred strains and crosses of sweet corn. Jour. Agr. Research, 53, 927–954.

McCulloch, L. 1918 A morphological and cultural note on the organism causing Stewart's disease of sweet corn. Phytopath., 8, 440–441.

Smith, E. F. 1898 Notes on Stewart's sweet corn germ, *Pseudomonas stewarti* n. sp. Amer. Assoc. Adv. Sci. Proc., 47, 422–426.

Smith, E. F. 1901 The cultural characters of *Pseudomonas hyacinthi, Ps. campestris, Ps. phaseoli*, and *Ps. stewarti*—four one-flagellate yellow bacteria parasitic on plants. United States Dept. Agr., Div. Veg. Physiol. and Path. Bul. 28.

Smith, E. F. 1914 Bacteria in Relation to Plant Diseases. Vol. 3, Washington, D. C. (Carnegie Inst. Wash. Pub. 27).

Stewart, F. C. 1897 A bacterial disease of sweet corn. New York State Agr. Exp. Sta. Bul., 130, 423–439.

Stiles, H. R., Peterson, W. H., and Fred, E. B. 1926 A rapid method for the determination of sugar in bacterial cultures. Jour. Bact., 12, 427–439.

Wellhausen, E. J. 1935 Genetic investigations of bacterial wilt resistance in corn as caused by *Bacterium stewarti* (Smith) Migula. Iowa State Col. Jour. Sci., 9, 539–547.

THE DETECTION OF ANTIGENIC VARIANTS OF BRUCELLA BY MEANS OF AN OPSONOCYTOPHAGIC TEST[1]

MYRTLE MUNGER AND I. FOREST HUDDLESON

Department of Bacteriology, Michigan State College

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The increasing employment of the *Brucella* opsonocytophagic test in conjunction with an allergic test (1934, 1937) as a means of determining *Brucella* immunity, infection and susceptibility in human beings, has necessitated a thorough study of a large number of cultures of *Brucella* to determine whether there exist strains unsuitable for the test.

It is common knowledge among those who have been concerned with *Brucella*, that antigenic variants make unreliable antigens for the agglutination test. The reliability of the test depends upon the employment of a normal strain. It was thought at one time that antigenic variants could be detected and eliminated by means of the thermo-agglutination test of Burnet (1928). More recent studies (Huddleson, Scales and Sorenson, 1936), however, conducted on a large number of strains for a period of 4 years have revealed that such a test could not be relied upon as a constant means for detecting variants. Very often, a strain found to be agglutinated by heat in one trial would pass through several successive trials before it again manifested this phenomenon.

Di Aichelburg (1934) has proposed a very unique and simple test for the detection of antigenic variants of *Brucella*. The test is made by suspending cells from an agar-slant growth in a

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1:2000 dilution of basic fuchsin in distilled water and incubating the suspension for 2 hours at 37°C. All unstable strains according to Di Aichelburg are agglutinated in varying degrees at the end of 2 hours, while normal ones remain uniformly suspended. Many of the antigenic variants are agglutinated within 5 minutes after being suspended in the dye solution. While the dye agglutination test is far superior to and much more delicate than the thermo-agglutination test for detecting antigenic variants, it too has not been found to give constant results with many known variants.

Preliminary studies of many cultures of the three species of *Brucella* revealed that certain strains were phagocytized in large numbers by the neutrophiles in whole citrated blood from normal individuals regardless of whether they had previously developed immune opsonins to *Brucella*. Previous studies have shown that leukocytes in whole citrated blood of non-immune or non-infected individuals phagocytize few if any organisms of a known normal strain.

Many of the strains which were phagocytized non-specifically were known to possess antigenic characteristics and behave to physical tests like the "para" forms of Nègre and Raynaud (1912) or the thermo-agglutinable forms of Burnet (1928). Normal strains usually develop into thermo-agglutinable or unstable antigenic variants when cultivated for any length of time in broth. Such variants have been termed the rough form of *Brucella*, but they in no manner conform to the characteristics of the rough form described by Mallmann and Gallo (1933) and recently confirmed by one of the authors.

In order to determine the accuracy and consistency of the thermo-agglutination test, the dye agglutination test and the opsonocytophagic test in detecting antigenic variants of *Brucella*, the three were conducted simultaneously with the growth from one liver-agar slant of each culture. The tests were repeated several times at wide intervals on normal strains and on strains that had on one occasion during the past 8 years been found to be thermo-agglutinable.

The thermo-agglutination test was made by suspending a por-

tion of the surface growth of the agar slant in physiological salt solution at pH 8. The turbidity of the suspension was 5 cm. by the Gates apparatus. The suspended cells were held in a water bath at 80°C. for 2 hours. The stability of the cells was noted at the end of the 2-hour period and after standing at room temperature for 15 hours.

The dye agglutination test was made by suspending a portion of the growth in a 1:4000 dilution of basic fuchsin in distilled water. The suspended cells were incubated in a 37°C. room for 15 hours, following which the stability of the suspended cells was noted.

The opsonocytophagic test was made by suspending a portion of the growth in physiological salt solution to a turbidity of 6 mm. by the Gates apparatus. Into clean, small glass vials, such as are used for the agglutination or Kahn test, are placed 0.1 cc. of whole blood and 0.1 cc. of the bacterial suspension. After shaking thoroughly, the vials are placed in a water bath for 30 minutes at 37°C. The tubes may be agitated lightly at five-minute intervals during the period of incubation. Considerable sedimentation of the blood cells takes place during the incubation period. Directly after removing the tubes from the water bath, a small amount of the cell suspension is removed by means of a finely drawn capillary pipette to which is attached a small rubber bulb. A drop of the cell suspension is placed at one end of a thoroughly cleaned and polished glass slide and drawn across the slide by placing the end edge of another slide in front of the drop and at such an angle that the spread thins out and terminates at or near the opposite end. The blood film should be dried as rapidly as possible to prevent shrinking of the leucocytes. Rapid drying may be obtained by placing the slides in front of a small electric fan.

The slides are stained by covering the spreads with 0.5 cc. of Hastings' stain (Hartman and Leddon Co., Philadelphia). After an exposure of 20 seconds, 1 cc. of distilled water, buffered to a pH of 6, is added to the stain on the slide and thoroughly mixed. At the end of 10 minutes, the spread is gently but thoroughly washed free from stain with distilled water and dried in front of an electric fan.

The *Brucella* opsonic activity of the blood is determined by examining spreads under an oil immersion objective with a 12× ocular. A total of 25 polymorphonuclear cells is examined in different sections of the spread, and each cell is grouped in one of the 5 following groups: −, when no phagocytosis occurs; 1+, when 25 per cent of the cells show phagocytosis; 2+, when 50 per cent of the cells show phagocytosis; 3+, when 75 per cent of the cells show phagocytosis; 4+, when 100 per cent of the cells show phagocytosis.

TABLE 1

Comparison of thermo-agglutination, dye-agglutination and phagocytosis of variant Brucella cultures

Culture		Thermo-agglutination			Dye-agglutination			Normal blood phagocytosis	
Number	Type	First test	Second test	Third test	First test	Second test	Third test	First test	Third test
S422	V	−	−	−	−	−	−	3+	1+
S429	V	2+	2+	4+	4+	4+	4+	3+	3+
S410	V	−	−	3+	−	−	3+	1+	3+
M623	V	−	−	2+	−	3+	2+	4+	3+
M350	V	−	−	2+	−	−	3+	4+	2+
M336	V	2+	−	4+	−	−	4+	2+	3+
A85	V	4+	4+	4+	4+	4+	4+	4+	4+
A36	V	4+	4+	4+	4+	4+	4+	4+	4+
A132	V	4+	4+	4+	4+	4+	4+	4+	4+
S400	N	−	−	−	−	−	−	−	−
M78	N	−	−	−	−	−	−	−	−
A154	N	−	−	−	−	−	−	−	−

+ = degree of agglutination or phagocytosis; V = variant culture; N = normal culture; S = *suis*; M = *melitensis*; A = *abortus*.

A total of 52 known antigenic variants and 9 normal strains were studied. Of 9 variants of *Brucella abortus*, only 6 were positive to either the dye or heat agglutination on one or more trials. Of 9 variants of *Brucella suis*, only 4 were positive to the physical tests on one or more trials. Of 34 variants of *Brucella melitensis*, only 21 were positive to the physical tests on one or more trials. All 52 strains were phagocytized in large numbers by cells in normal blood on all trials. All normal strains were

phagocytized to only a slight degree if at all by cells in normal blood.

The comparative results of a series of three trials on 9 variants and three normal strains are presented in table 1. The recorded data show that there is no consistency in the results of either the thermo-agglutination or dye agglutination test. The results of the phagocytic test conducted with normal blood agreed with the previous history of the cultures. The degree of phagocytosis observed with a given variant culture varied considerably in the repeated tests. In no instance, however, was the degree of phagocytosis as low as that observed with normal cultures and normal blood.

If, in the examination of a given culture of *Brucella* by means of the phagocytic test, it is found that 25 per cent or more of the neutrophiles in a normal whole blood from human beings or guinea pigs show ingestion of bacteria, the culture contains variant cells and is not suitable for phagocytic or agglutination studies.

SUMMARY

The most satisfactory method for detecting an antigenic variant culture of *Brucella* is an opsonocytophagic test conducted with citrated normal whole blood from humans or guinea pigs.

The bacterial cells of an antigenic variant culture are phagocytized in large numbers by the polymorphonuclear cells in citrated normal whole blood from humans and guinea pigs. Bacterial cells of a normal culture are phagocytized, slightly if at all, by leukocytes in citrated normal blood of the same species.

Antigenic variants of *Brucella* are unsuitable for use in the opsonocytophagic test for detecting specific *Brucella* opsonins in blood of human beings or animals.

REFERENCES

DI AICHELBURG, U. 1934 Agglutination aspécifique avec la fuchsine basique dans les microbes du groupe *Brucella*. Boll. d. sez. ital., 6, 30–32.

BURNET, ET. 1928 La thermo-agglutination et l'évolution de l'espèce *Brucella*. Arch. Inst. Pasteur de Tunis, 17, 128–146.

HUDDLESON, I. FOREST. 1934 *Brucella* Infections in Animals and Man. Published by the Commonwealth Fund.

HUDDLESON, I. F., GOULD, S. E., MUNGER, MYRTLE, AND POLSON DORIS. 1937 A study of *Brucella* infection and immunity in humans. Submitted for publication in Amer. Jour. Trop. Med. and Hyg.

HUDDLESON, I. FOREST, SCALES, J. W., AND SORENSON, O. J. 1936 Non-specific agglutination in the *Brucella* group. Tec. Bul. No. 149, Mich. Agr. Exp. Sta.

MALLMAN, W. L., AND GALLO, F. 1933 Studies on the dissociation of the *Brucella* groups. Jour. Agr. Res., 46, 267–279.

NÈGRE, L., AND M. RAYNAUD 1912 Identification des paramelitensis par l'épreuve de la saturation des agglutinines. Compt. rend. Soc. de biol., 72, 1052–1054.

COLONY ORGANIZATION OF CERTAIN BACTERIA WITH REFERENCE TO SPORULATION[1]

H. C. GREENE

Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wisconsin

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Accounts of the study of colonial organization of bacteria and other microörganisms by means of thin sections have appeared sporadically in the literature for nearly fifty years.

Neisser in 1888 devised a procedure for obtaining individual sections of colonies grown in gelatin or agar shake cultures. The plugs of medium were removed from the culture tubes by gentle warming, cleared in a 1 per cent aqueous solution of potassium bichromate, washed, and carried through a graded series of alcohols into 95 per cent alcohol. The plugs were cut into blocks with one colony in each. The blocks were then mounted on pieces of cork, with gum arabic, and placed in absolute alcohol for 24 hours. By means of a sliding microtome, sections were cut, mounted on slides with fixative, stained, and cleared in the usual manner. Neisser's paper is confined to a description of the technique alone, but is of interest since it is apparently the first comprehensive report of a method for sectioning colonies of microörganisms.

Hutchinson in 1907 studied sections of surface and subsurface colonies of *Bacillus subtilis*, *Bacillus megatherium*, *Saccharomyces cerevisiae*, "*Mycoderma*" *cerevisiae*, and *Oidium lactis*. In the case of surface colonies, he poured lukewarm melted agar containing 3 per cent formalin over the colonies, and, when the agar hardened, removed the colonies intact. The colonies were then imbedded in paraffin and sectioned. In the bacterial colonies he found the cells at the margin to be of normal size and ap-

[1] This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

pearance, while involution forms occurred in the center. In surface colonies spore formation was most profuse at the upper surface. Yeast colonies were more complicated in organization than bacterial colonies. Round or oval cells were found at the colony surface, while the cells next to the substrate were slender and elongated. In some cases an extensive mycelial system developed in the substrate. Mycelium was more abundantly produced in old cultures where the medium presumably contained an abundance of growth products. Hutchinson's extensive paper contains diagrams and photographs of colonies in section, as well as a complete discussion and bibliography of the relevant work of other investigators.

Zikes in 1916 studied colonies of *Schizosaccharomyces pombe*, *Schizosaccharomyces mellacei*, *Zygosaccharomyces priorianus*, and also certain bacteria (species not given) isolated from beer wort. He observed abnormally elevated colonies of these organisms on plates containing a high percentage of gelatin and incubated at a low temperature. Zikes prepared sections from colonies in celloidin. He found that elongated cells predominated at the base of the colonies, but that in the upper portions the cells were progressively smaller. The difference in cell size is ascribed to unequal nutritive conditions.

Legroux and Magrou in 1920 studied wrinkled, atypical colonies of the cholera vibrio in section. By applying differential stains they demonstrated a definite zonation in the complicated internal organization of the wrinkled colonies. The vibrios were localized in well-defined layers and zones, following the outlines of the colony sinuosities.

Truffaut and Bezssonoff (1922) observed on plating soil samples under aerobic conditions oval subsurface colonies which contained *Clostridium pastorianum* in association with aerobic bacilli. Sections showed a central core of sporulating *C. pastorianum* cells surrounded by a layer of aerobic bacilli. The predominant bacillus was gram-negative, and with the gram stain the authors obtained good differentiation between the central mass of gram-positive *C. pastorianum* cells and the overlying bacilli.

Kahn and Nonidez (1936), studying colonies of the tubercle

bacillus, used not only the standard paraffin method, but also the frozen section method and a combined colloidin-paraffin technique.

This paper presents a study of the relation of sporulation to the colony organization of three species of bacteria: *Clostridium acetobutylicum*, *Clostridium pasteurianum*, obligate anaerobes, and *Bacillus acetoethylicum*, a facultative anaerobe. Stock cultures of these bacteria are maintained in the collection of the Department of Agricultural Bacteriology at the University of Wisconsin.

The aim of this study was (*a*) to follow the course of sporulation in the colony by using a standard medium and a constant incubation temperature combined with varied growth times, and (*b*) in the case of *B. acetoethylicum* to study also the effect of certain environmental conditions on sporulation in the colony.

Since the results indicate that the sectioning technique may be used in further investigations, it is planned to extend this study of colony organization.

PROCEDURE

Inoculum consisted of young, actively growing cultures, containing few or no spores. Usually both surface and subsurface colonies were obtained by ordinary dilution methods. In some cases satisfactory surface colonies could be produced only by inoculating solidified medium with a tiny loop.

The following medium (liter basis) was used as standard in the time-sporulation series: Glucose 5.0 grams, beef extract 3.0 grams, peptone (Parke, Davis & Co. "Bacteriologic") 5.0 grams, agar 15.0 grams, tap water to make to 1 liter. The medium was adjusted to neutrality with NaOH before sterilization. For *C. acetobutylicum* 10 grams of glucose was used since that amount was found to be more favorable for sporulation. All results reported are based on colonies grown at 37°C.

Subsurface colonies were removed in the agar in which they developed. For purposes of orientation a thin layer of agar was poured over the surface of the medum above the colony before removal. The form of surface colonies was preserved by imbedding them under a layer of agar so that they could be removed

intact. As a rule, six to eight separately imbedded colonies constituted a sample. If more colonies were desired additional samples were taken. Colonies were fixed in formalin-acetic-alcohol or, where the medium contained excess $CaCO_3$, in 70 per cent alcohol only, and after dehydration, were imbedded in paraffin. Rapid and satisfactory dehydration was obtained with an alcohol-anilin-oil-of-wintergreen schedule.

A total of approximately 250 colonies were investigated. Sections of 3 μ or 5 μ thickness have usually been most satisfactory.

Two spore stains were tried, a modification of Dorner's with Ziehl's carbol fuchsin and nigrosin, and Conklin's modification of the Wirtz mercurochrome malachite-green stain. Neither was suitable since it proved very difficult to decolorize the colony matrix adequately after staining. Methylene blue, gentian violet, safranin, and Ziehl's carbol fuchsin were not satisfactory.

Nicolle's carbol thionin has been almost exclusively used. For staining, the stock solution (0.25 per cent thionin in 50 per cent alcohol) is diluted with an equal volume of 2 per cent phenol. The stain is used at room temperature, and 3 to 5 seconds exposure is sufficient.

Stained mounts were protected with thin films of rapidly drying "Vinylite A" in butyl acetate (Skiles and Georgi, 1937). The "Vinylite" solution is run over the mount, the excess drained off, and a very thin, transparent film remains, giving a permanent mount after an hour's drying.

RESULTS

Clostridium acetobutylicum. Vegetative cells stain uniformly, young clostridia stain deeply at one end, while clostridia containing mature spores, and free spores, do not stain appreciably with thionin.

In subsurface colonies sporulation is apparently initiated at the periphery of the colony and progresses inward (plate 1, A).

In surface colonies observations were made at time periods ranging from 40 to 144 hours, but sporulation has never been noted under 60 hours. Transitional stages showing the young

clostridia immediately preceding sporulation are found only with difficulty. Sporulation is initiated throughout the central and lower portion (that portion next the agar substrate) of the colony. The center portions of older surface colonies show the greatest development of mature spores (plate 1, B), while there is a transition toward the margins from young clostridia to vegetative cells at the extreme outside edges of the colonies.

Dark-field illumination is very useful in giving a clear picture, under the lower powers, of the total extent of sporulation in older colonies of *C. acetobutylicum*, since the highly refractive spores are so imbedded in the colony matrix that it is difficult to discern them with ordinary light, and then only in the restricted oil immersion field (fig. 1).

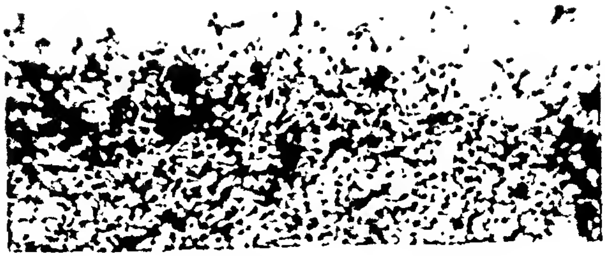
Clostridium pasteurianum. Vegetative cells usually stain uniformly, but have been observed with a pronounced banding. Young clostridia stain deeply at one end, while clostridia containing mature spores, and free spores, do not stain appreciably with thionin.

Subsurface colonies were fixed at representative time periods ranging from 29 through 144 hours. At 29 hours vegetative cells only are present. At 40 hours there is a mixture of vegetative cells and young clostridia, while at 60 hours clostridia with mature spores, and free spores, are also present. At 144 hours young clostridia are not found (plate 1, C, D). A few vegetative cells are always found, even in the most highly sporulated colonies. It is doubtful whether there is a fixed sporing pattern, but it appears that at certain time periods certain types of cells predominate throughout the colony, but are not noticeably localized.

Surface colonies were fixed at time periods ranging from 44 through 144 hours. According to colony age, zones can be established where certain cell types predominate. Thus, in a 44-hour colony young, terminally-staining clostridia predominate in the central portion (fig. 2) while vegetative cells occur in progressively increasing numbers toward the colony margins, where vegetative cells only are found. In a 64-hour colony (plate 1, E) mature clostridia and free spores occur profusely in the center

Fig. 1.

a



b

Fig. 2 a b

Fig. 3

FIGS. 1-3

(CC); midway between the center and the margins young clostridia are most abundant (BC); and at the extreme margins vegetative cells only are present (AB).

Bacillus acetoethylicum. Vegetative cells stain uniformly. Young clostridia stain deeply at one end. This organism is especially favorable for study by the sectioning technique since the colonies, while only moderately compact, section well, and since the conspicuous spores soon lie free and take a pale reddish-purple differential stain with thionin.

Sporulation in the case of *B. acetoethylicum* is strongly favored by aerobic incubation, and has been observed only once under anaerobic conditions.

On the standard medium, the transitional stages leading to sporulation are initiated at about 40 hours. Mature spores appear at from 50 hours onward, and soon lie free within the colony. It is difficult to demonstrate mature spores still within the cells.

In subsurface colonies sporulation is very meagre and spores are found only at the colony periphery (plate 2, A). Experiments with oxidized media failed to produce changes in the manifestations of sporulation in subsurface colonies, under either aerobic or anaerobic conditions.

Surface colonies on dilution plates are often, if not always, initiated subsurface, and then break through, spreading out from a dense central zone. In this connection Orsos (1910), who studied colonial development in various bacteria and yeasts, found that the majority of surface colonies develop from subsurface colonies which form the "nucleus" of the resultant surface colony. In *B. acetoethylicum* the "nucleus" stains a light, uni-

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FIG. 1. CL. ACETOBUTYLICUM. PHOTOMICROGRAPH, DARK FIELD ILLUMINATION
Profuse sporulation in basal portion of a 90-hour surface colony. ×400

FIG. 2. CL. PASTEURIANUM. PHOTOMICROGRAPHS

A. Sector from top central portion of young 44-hour surface colony, showing young clostridia. ×700.

B. Section through entire central portion of same colony showing young clostridia predominating to the practical exclusion of other cell forms.

FIG. 3. B. ACETOETHYLICUM. PHOTOMICROGRAPH
Sector from colony surface showing profuse, localized sporulation. ×850

form blue with thionin, as opposed to the deeper purplish-b[illegible] of the rest of the colony. Such colonies are hence characterized by subsurface growth lobes which may be of greater volume than the surface portion (plate 2, E).

Investigation of the effect of varying environmental conditions on sporulation consisted in (*a*) use of medium with excess of $CaCO_3$, as opposed to medium without such addition, (*b*) varying agar concentrations from 1.5 to 3.0 per cent, (*c*) varying glucose concentrations from 0.1 to 2.5 per cent, and (*d*) use of aerobic as opposed to anaerobic conditions. These variables were used in diverse combinations. As a rule, growth periods were from 72 to 85 hours.

Findings concerning the influence of varying conditions on sporulation in aerobic surface colonies are as follows:

Media containing an excess of $CaCO_3$ strongly favor profuse and generalized sporulation without regard to the concentrations of the other constituents of the media. The effect of the excess $CaCO_3$ appears to be due, in part at least, to regulation of acid conditions unfavorable to sporulation which are created by the organism in the course of growth, particularly on media with 2.0 per cent or more of glucose (plate 2, B).

The remaining findings for aerobic surface colonies have to do with those produced on media which did not contain excess $CaCO_3$.

In the early stages of this study plates with unglazed porous clay tops were used. When such plates are incubated anaerobically there is no excessive moisture loss through the porous tops. However, when they are incubated aerobically at 37°C. the medium dries down rapidly with resultant concentration of the agar, which is present in the fairly high initial concentration of 1.5 per cent. In ordinary glass top plates the medium is not thus rapidly concentrated. These facts are of significance in that where media do not contain excess $CaCO_3$, differences in agar concentration appear to be correlated with sporulation variations, as detailed below.

Sporulation is not profuse nor especially localized when low

agar concentration is combined with low glucose concentration. It is even less profuse in the presence of low agar and high glucose concentration (2.0 per cent or more), where the factor of acidity seemingly enters (plate 2, C). The same holds to a lesser degree for high agar in combination with high glucose concentration.

Sporulation is more profuse and also tends to be localized at the colony surface when there is a high agar concentration combined with a relatively low glucose concentration (fig. 3) (plate 1, F; plate 2, D, E).

Thus, in glass top plates, sporulation is not profuse or localized when the initial agar concentration is 1.5 per cent combined with 0.5 per cent glucose. However, with the same medium in clay top plates sporulation is more profuse and localized at the colony surface. On the other hand, with an initial agar concentration of 3.0 per cent, combined with 0.5 per cent glucose in glass top plates, sporulation is profuse and localized, as is also true for the same combination in clay top plates.

Under anaerobic conditions sporulation has not been observed in subsurface colonies. In surface colonies grown anaerobically sporulation has been observed once only. The series included media with and without excess $CaCO_3$, combined with high and with low agar concentrations. In all cases sporulation was very slight with no apparent localization.

SUMMARY

1. Stained sections of colonies of *Clostridium acetobutylicum*, *Clostridium pasteurianum*, and *Bacillus acetoethylicum* were studied to determine the course of sporulation within the colonies under (*a*) conditions taken as standard, and (*b*) in *Bacillus acetoethylicum* under varying as well as standard conditions.

2. In *Clostridium acetobutylicum* subsurface colonies are sporulated principally at the colony periphery. In surface colonies the spores are not found at the upper surface of the colony, but are confined to the central and basal portions.

3. In *Clostridium pasteurianum* subsurface colonies become profusely sporulated, but do not show a regular pattern of spor-

ulation. The older surface colonies show a transition from vegetative cells at the colony margins, to young clostridia in the adjacent portions, to mature spores in the middle region.

4. In *Bacillus acetoethylicum* sporulation is significantly profuse only under aerobic conditions. Subsurface colonies are sporulated only at the colony periphery. In surface colonies sporulation is most profuse and tends to be localized at the upper surface of the colony when a relatively high agar concentration is combined with a relatively low glucose concentration. In surface colonies on media containing excess $CaCO_3$ sporulation tends to be profuse, but ceases to be localized regardless of the concentrations of the other constituents of the medium.

REFERENCES

HUTCHINSON, H. B. 1907 Ueber Form und Bau der Kolonien niederer Pilze. Centbl. Bakt. II, 17, 417–427, 593–604.

KAHN, M. C., AND NONIDEZ, J. F. 1936 The rôle of non-acid-fast rods and granules in the developmental cycle of the tubercle bacillus. Amer. Rev. Tuberc., 34, 361–382.

LEGROUX, R., AND MAGROU, J. 1920 État organisé des colonies bactériennes. Ann. Inst. Pasteur, 34, 417–431.

NEISSER, A. 1888 Kleine Beiträge zur bakterioskopischen Methodik. I. Mikroskopische Schnittpräparate aus Reagensglaskulturen. Centbl. Bakt., 3, 506–510.

ORSOS, F. 1910 Die Form der tiefliegenden Bakterien- und Hefekolonien. Centbl. Bakt. I. Orig., 54, 289–328.

SKILES, B. F., AND GEORGI, C. E. 1937 The use of synthetic resins in the preparation of permanent bacterial mounts. Science, 85, 367.

TRUFFAUT, G., AND BEZSSONOFF, N. 1922 La stérilization partielle ou désinfection du sol. Ses effets et leurs causes. Sci. Sol. (Truffaut). 1, 3–61.

ZIKES, H. 1916 Über abnorme Kolonienbildungen bei Hefen und Bakterien Centbl. Bakt. II, 46, 1–4

PLATE I

A. *Cl. acetobutylicum*. Subsurface colony, sporulated at periphery. ×30

B. *Cl. acetobutylicum*. Surface colony, sporulated basally. ×40.

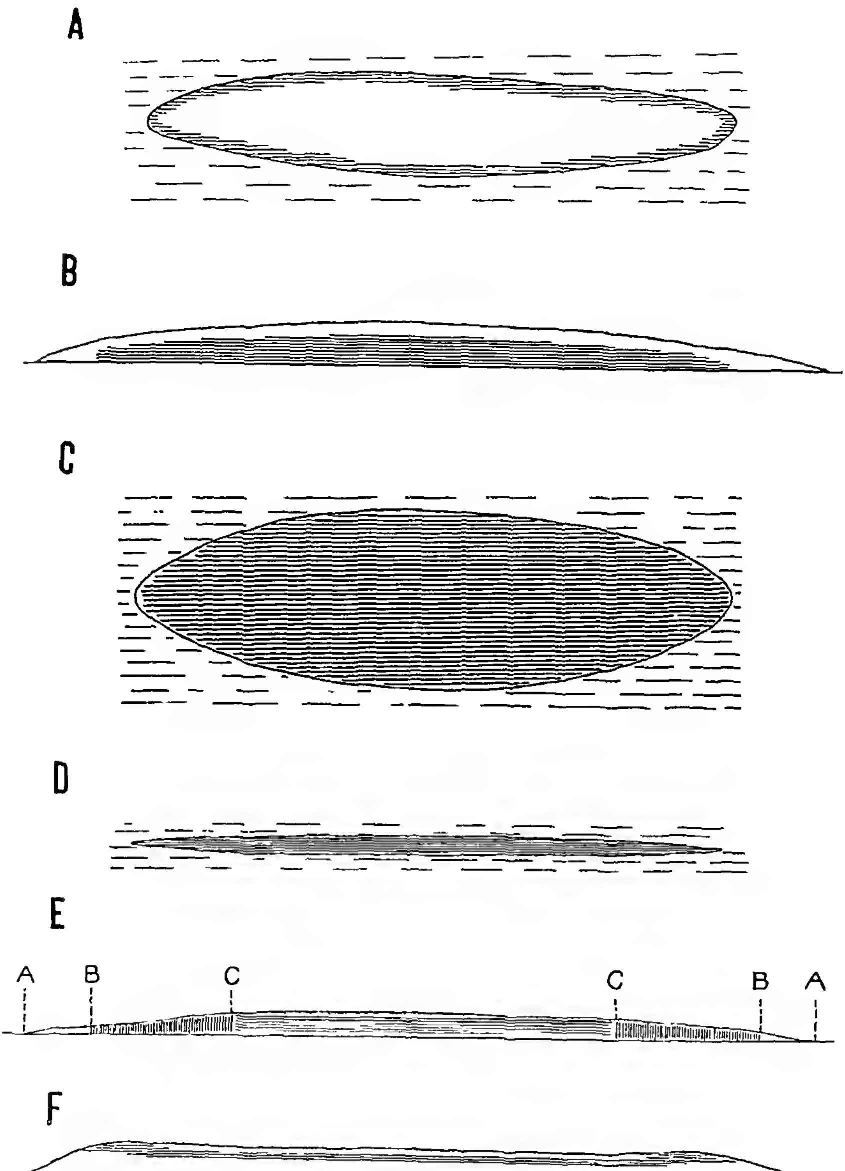
C. *Cl. pasteurianum*. Subsurface colony, oval, sporulated throughout ×125.

D. *Cl. pasteurianum*. Subsurface colony, slender lens-shaped, sporulated throughout. ×60.

E. *Cl. pasteurianum*. Surface colony showing zones of cell types ×20

F. *B. acetoethylicum*. Surface colony. Sporulation localized. ×30.

Diagrams were drawn from actual colonies.



(H. C. Greene: Colony Organization and Sporulation)

PLATE II

A *B. acetoethylicum* Sub-surface colony, slight sporulation at periphery ×125.

B *B. acetoethylicum* Surface colony, sporulated throughout, grown on medium with excess $CaCO_3$. ×60

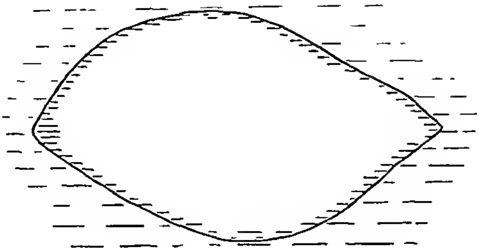
C. *B. acetoethylicum* Surface colony, sporulation slight, not definitely localized ×45.

D. *B. acetoethylicum* Surface colony, sporulation profuse and localized ×40

E *B. acetoethylicum* Surface colony similar to D, but with a large subsurface lobe. ×60

Diagrams were drawn from actual colonies

A



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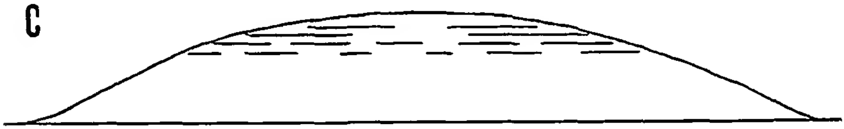

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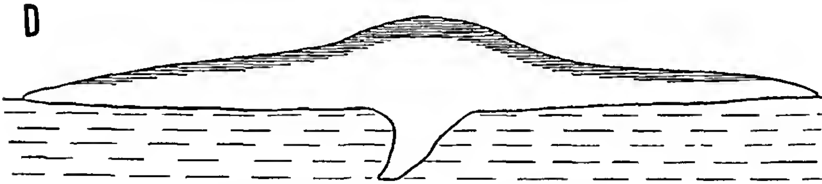

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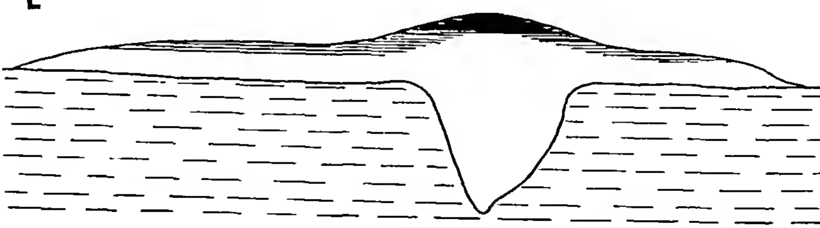

(H. C. Greene: Colony Organization and Sporulation)

THE OCCURRENCE AND CHARACTERISTICS OF CHITINOCLASTIC BACTERIA IN THE SEA

CLAUDE E. ZOBELL AND SYDNEY C. RITTENBERG

Scripps Institution of Oceanography, University of California, La Jolla

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Chitin is a tough leathery insoluble substance of indeterminate chemical structure somewhat resembling cellulose. It is generally believed to be a polymer of glucosamine in which each amino group is acetylated. Chitin is the chief constituent of the exoskeleton of Arthropods and it occurs in some Mollusks, Coelenterates and Protozoa as well as in certain fungi. There may be more than one kind of chitin but the observations of Diehl (1936), van Iterson *et al.* (1936) and others indicate that animal and fungoid chitins are identical.

Large quantities of chitin are produced in the oceans of the world each year. From data given by Johnstone (1908) on the abundance of Copepods and their chitin content it is estimated that just this one sub-class of planktonic crustacea, some of which form 10 to 12 chitinous casts in their development stages, produces several billion tons of chitin annually. Most of this is probably utilized by biological agents because little accumulates in marine sediments and moreover, if it were not decomposed, it would soon become a serious drain upon carbon and nitrogen in the cycle of these elements.

The fragmentary and contradictory literature on the subject fails to indicate to what extent chitin may be utilized as a source of food by animals which may ingest it. Some workers claim to have detected chitinase in the alimentary tract of certain animals while other investigators find no evidence that animals can digest chitin. However, the possibility of symbiotic bacteria aiding animals in the digestion of chitin should not be overlooked.

Bacteria are probably responsible for the disintegration of much chitin in the sea. Bertel (1935), Bucherer (1935), Waksman *et al.* (1933), ZoBell and Anderson (1936) and others have reported chitin digestion by mixed cultures from marine sources but have not described the bacteria involved. These observations, together with those of Stuart (1936) on the occurrence of halophilic chitinovorous bacteria in marine salt from Africa, S. America, Spain, California and the West Indies, indicate a worldwide distribution of chitin-digesting bacteria in the sea. It is doubtful if *Bacillus chitinovorus* which Benecke (1905) isolated from Kiel harbor, where there is much terrigenous contamination and dilution of the harbor water, is a true marine species, particularly in view of the fact that Rammelberg (1931) isolated an identical organism from soil. Chitinovors from terrestrial sources have been reported by Rammelberg (1931), Folpmers (1921), Jensen (1932), Johnson (1932) and others.

This paper is concerned primarily with the demonstration of chitinoclastic bacteria in the sea and a study of their physiological characteristics. The term *chitinoclastic* is applied to bacteria which in any way attack the chitin molecule. It includes the so-called *chitinovors* besides others revealed by these studies which split certain radicals or side-chains from the chitin molecule although they do not *devour* chitin, either in the sense of deriving nutrients from it or causing its dissolution.

EXPERIMENTAL METHODS

Chitinoclastic bacteria were detected by inoculating chitin medium with samples of raw sea water, bottom sediments and other marine materials. The chitin medium was prepared by partly covering 1 x 5 cm. strips of purified chitin in test tubes with sea water and sterilizing at 124°C. for 20 minutes. The chitin strips were prepared from lobster shells by methods similar to those used by Benton (1935). After successive prolonged treatments with 1 per cent hydrochloric acid, 2 per cent potassium hydroxide, several changes of boiling alcohol and finally water, the chitin was colorless and reacted positively to the tests for chitin listed by Buchanan and Fulmer (1928). Simple

nitrogen and carbon compounds were added to some of the media as sources of readily available nutrients. All media were prepared with sea water.

The inoculated media were incubated at 21°C. and examined periodically for evidence of chitinoclastic activity. The visible dissolution of chitin, the liberation of ammonia, acids or reducing sugars from chitin, or the growth of bacteria in the medium consisting of only chitin and sea water were used as criteria of chitinoclastic activity. In some of the inoculated media such activity became manifest within two or three days but, in others, not until the cultures had been incubated five or six months. In the preliminary work all cultures were held at least six months before being discarded as negative. It was found that two months' incubation was adequate to detect most chitinoclastic species.

Pure cultures of chitinoclastic bacteria were obtained by inoculating a second tube of chitin medium with a loopful of material from the positive primary raw cultures. Growth from the enriched cultures was then streaked on nutrient agar plates. The different types of colonies which developed were isolated, retested for their ability to digest chitin and checked for purity by examining stained smears and colony characteristics. About one-fourth of the pure cultures thus isolated proved to be chitinoclastic.

OCCURRENCE IN THE SEA

In preliminary surveys, chitinoclastic bacteria were demonstrated in nearly all 5- to 6-gram portions of bottom sediments which were inoculated into chitin media. Included in the survey were several sediment samples collected from the beach or shallow water along the coast of southern California. Many others were collected at stations occupied by the boat SCRIPPS, some of which were nearly 200 miles from the mainland and from water depths as great as 2000 meters.

Estimating their relative abundance by the minimum dilution method, chitinoclastic bacteria were found to be very unevenly distributed in bottom sediments. For example, a given sample may contain several hundred chitinoclasts per gram while in a similar sample immediately adjacent there may be less than

one per gram. This lack of uniformity in the distribution of bacteria within a limited area has been discussed by ZoBell and Anderson (1936) who attribute it to the unevenness in the distribution of nutrient particles and to the tendency of bacteria to colonize.

Chitinoclasts are most numerous in the topmost layers of mud where as many as a thousand per gram have been found. The number decreases sharply with core depth although some have been recovered from the bottom of mud cores exceeding 60 cm. in length. No relationships were noted between the abundance of chitinoclastic bacteria in sediments and the depth of the overlying water or the distance from the mainland. The largest chitinoclastic populations were usually associated with coarser sedimentary materials like sand, with which the chitinous particles are concentrated by the assorting action of the forces of sedimentation.

Between 0.1 and 1.0 per cent of the bacteria found in sea water are chitinoclastic to some degree, the total bacterial population of the topmost 25 to 50 meters of water being from a few to a few thousand per cubic centimeter. Below this depth the number of bacteria in the water drops off sharply to 200 meters, below which, only occasionally, can bacteria be demonstrated at all except in the immediate proximity of the bottom. Following initial isolation most of the bacteria will grow in sea water media but not in corresponding fresh-water media which, together with the fact that they have been isolated at places remote from possibilities of terrestrial contamination, is regarded as evidence that they are species which are indigenous to a marine environment.

The richest source of chitinoclastic bacteria is the remains of decomposing crabs, lobsters or other crustacea found on the bottom or along the sea shore. Thirteen different kinds of chitinoclastic bacteria have been isolated from this source. Johnson (1932) isolated several kinds of chitin-destroying bacteria from the shells of fresh-water crabs undergoing decomposition, and recently Hess (1937) recovered several strains of chitin digesters from living lobsters having a shell disease.

From a hundred to more than a thousand chitinoclastic bacteria per cubic centimeter were found in the stomach contents of squid and other cephalopods which ingest chitinous food. This suggests the possibility that such bacteria may play an important rôle as symbionts which aid animals in the digestion of chitin. Benton (1935) recovered chitinoclastic bacteria from the intestines of fishes, frogs and bats.

Chitinoclastic activity was exhibited by 12 of the 85 pure cultures which constitute the Scripps Institution stock collection and which are believed to be representative of the aerobic heterotrophs inhabiting the sea. The cultures have been isolated at random during a period of several years from various marine materials without deference to any special physiological function. This observation gives further evidence of the widespread distribution of chitinoclastic bacteria in the sea, and since the cultures have been cultivated for several years in the absence of chitin, it shows that chitinoclastic ability is not limited to bacteria which have been cultivated in the presence of chitin.

CHARACTERISTICS OF MARINE CHITINOCLASTS

To date, 31 different pure cultures of chitinoclastic bacteria have been isolated from various marine materials. Several were isolated from two or more sources thereby indicating a widespread distribution of these species. While all of them are believed to be new and undescribed species, they have not been studied completely enough to warrant classifying them as such, and even if all of the important characteristics had been determined, the authors would hesitate to assign generic names to the organisms in the face of the present chaotic condition of systematic bacteriology.

The problem is further complicated by the fact that nearly all of these bacteria grow either preferentially or exclusively in sea water media. Therefore in characterizing the bacteria it is usually necessary to deviate from Standard Method procedures to the extent of substituting sea water for fresh water in the differential media. Few of the marine species show any growth on the conventional potato and milk media, which are still widely

used to characterize bacteria in spite of their highly variable composition. Following prolonged laboratory cultivation or acclimatization procedures in which the salt content of the media is gradually reduced with each successive transfer, most of the cultures slowly develop the ability to grow in fresh-water media but it is not known how many other characteristics are changed during the adaptation process. It has been noted that the tendency to produce pigment is lessened, the temperature range of growth is widened and the ability to liquefy agar is lost during such acclimatization.

Table 1 gives the morphological and physiological characteristics of 14 representative chitinoclastic bacteria most of which have been recovered two or more times from various marine materials. Gram-negative, slender to ovoid rods varying only slightly in size and shape predominate. Only one chitinoclastic coccus and two species of vibrio have been found to date. Neither staining procedures nor thermotolerance tests indicated endospore formation in any of the cultures. Flagellation was not determined, but all except four of the 31 cultures were found to be actively motile. Encapsulation is a common property of the chitinoclasts but one which is highly variable under different conditions of cultivation. Involutionary forms appeared in most of the cultures, their abundance increasing with the age of the cultures. Chains of four or more cells were noted in none of the cultures and pairs occurred only infrequently.

In view of the structural similarity of chitin and cellulose it is noteworthy that none of the chitinoclastic bacteria digest cellulose. With few exceptions they are only feebly saccharolytic, nearly half of them failing to ferment any of the simple sugars and none forming gas from carbohydrates. Although chitin is probably a glucosamine, many of the chitinoclasts are unable to utilize glucose. Interestingly enough, some of them which attack neither glucose, maltose, sucrose nor lactose hydrolyze starch with acid production. Most of the pure cultures are actively proteolytic as indicated by their ability to liquefy gelatin and to produce ammonia from various proteinaceous substrates.

The chitinoclasts differ greatly in their nitrogen and carbon or

TABLE 1

Morphological and physiological characteristics of sixteen representative marine chitinoclastic bacteria as determined in sea water media

CULTURE NUMBER	AVERAGE SIZE	GRAM STAIN	MOTILITY	PIGMENTATION ON CHITIN MEDIUM	GLUCOSE	LACTOSE	SUCROSE	GLYCEROL	MANNITOL	STARCH HYDROLYSIS	CELLULOSE DIGESTION	GELATIN LIQUEFACTION	H_2S PRODUCTION	AMMONIA UTILIZATION	ACETATE UTILIZATION	NITRATE REDUCTION	MATERIAL FROM WHICH ISOLATED*
	microns																
2	2.1 x 0.6	—	—	Yellow	A	—	—	—	—	+	—	+	+	+	+	—	3, 5
3	3.2 x 1.3	—	—	None	—	—	A	—	—	+	—	+	—	+	+	—	2, 3
6	2.4 x 1.0	—	—	None	A	—	A	A	A	+	—	+	+	+	+	+	1, 2, 3, 4, 5
18	1.5 x 0.4	—	—	Yellow	—	—	—	—	—	+	—	+	+	+	+	—	2, 3, 4, 5
24	1.2 x 0.4	—	—	None	—	—	—	A	—	+	—	—	—	—	—	+	2, 3
29	3.0 x 0.6	—	—	Orange	—	—	A	—	—	—	—	+	+	+	+	—	1, 2
32	3.2 x 1.4	—	—	None	A	—	A	—	A	+	—	+	—	+	+	—	1, 3, 5
34	1.7 x 0.9	—	—	Yellow	—	A	—	—	—	+	—	+	—	+	+	—	1, 4
37	2.0 x 0.5	—	—	Pink	—	—	A	—	—	—	—	+	—	+	+	+	4, 5
47	0.8 x 0.6	—	—	Brown	A	—	—	A	A	—	—	+	—	+	+	+	3, 4, 5
50	1.4 x 1.1	—	—	Yellow	—	—	—	—	—	—	—	—	+	+	+	—	2, 3, 4
71	2.5 x 0.5	—	—	None	A	—	—	—	—	+	—	—	+	—	—	—	1, 2, 3, 4, 5
75	3.9 x 0.4	—	—	Brown	—	—	—	—	—	+	—	+	—	+	+	—	3
77	2.9 x 0.6	—	—	Brown	—	—	A	—	—	—	—	+	—	+	+	+	4, 5

* 1 = Bottom sediment; 2 = Crustacea; 3 = Squid stomach; 4 = Sea water; 5 = Tide pools.
A = Acid production.

energy requirements as well as in their action on chitin. Some multiply freely in a medium consisting of only pure chitin in sea water. They may not otherwise detectably effect the chitin, they may dissolve it, or their growth may be accompanied by the liberation of ammonia (table 2) or other decomposition products. Another group of chitinoclasts can derive their nitrogen from chitin when an available carbon or energy source such as the salt of an organic acid or a simple carbohydrate is added, after which some of them may merely multiply without otherwise detectably effecting the chitin while others cause its dissolution. A third

TABLE 2

Liberation of ammonia from chitin medium by representative chitinoclastic bacteria after varying periods of incubation at 21°C. and the elapsed time before there was any visible disintegration of the chitin

CULTURE NUMBER	AMMONIA REACTION AFTER				CHITIN DISINTEGRATION VISIBLE AFTER
	2 days	4 days	9 days	16 days	
5	−	−	++	+++	None after 5 months
6	−	−	+	++	28 days
8	−	+	+	+++	9 days
9	−	−	+	++	None after 5 months
10	−	+	++	+++	18 days
18	−	+	++	+++	10 days
28	−	−	+	+++	13 days
37	−	+	++	+++	31 days
63	+	++	+++	++++	10 days
Control	−	−	−	−	None after 5 months

type has been isolated from the sea which attacks chitin only in the presence of both carbon and nitrogen nutrients such as peptone. Further studies must determine whether the soluble decomposition products are inadequate for the carbon and nitrogen nutrition of such cultures or if they produce a chitinase only after multiplying for some time in the presence of chitin. More credence is placed in the former explanation in view of the fact that some of the stock cultures referred to above which have been cultivated on nutrient agar for several years started to attack the chitin within two or three days after being transferred to a chitin medium.

Observations on certain mixed cultures indicate that there are also symbiotic relationships in which two or more bacteria together can attack chitin although neither alone is endowed with this property. The investigation has revealed no obligate chitinovors, or bacteria which require chitin. The presence of peptone and other simple nutrients in chitin medium accelerates the chitinoclastic activity of most bacteria probably due to the growth-promoting properties of the peptone.

No pure cultures have been observed which can obtain their energy and carbon but not their nitrogen requirements from

TABLE 3

pH of sea water-chitin medium inoculated with chitinoclastic bacteria after different periods of incubation at 21°C. as measured with a glass electrode and Beckman pH meter

CULTURE NUMBER	INITIAL pH	pH OF MEDIUM AFTER				CHITIN DISINTEGRATION VISIBLE AFTER
		4 days	8 days	16 days	33 days	
8	7.5	7.3	6.9	6.7*	5.5	9 days
14	7.6	7.6	7.2	6.7	7.0	None after 5 months
17	7.6	7.3	7.0	6.4*	5.3	11 days
18	7.5	7.5	7.4	7.6	7.8	19 days
28	7.5	7.3	6.9	5.9*	5.0	13 days
37	7.5	7.3	7.0	6.6	6.0*	28 days
56	7.5	7.7	7.5	7.6	7.7	33 days
63	7.5	7.5	7.4	7.6	7.9	16 days
Control	7.6	7.6	7.7	7.7	7.6	None after 5 months

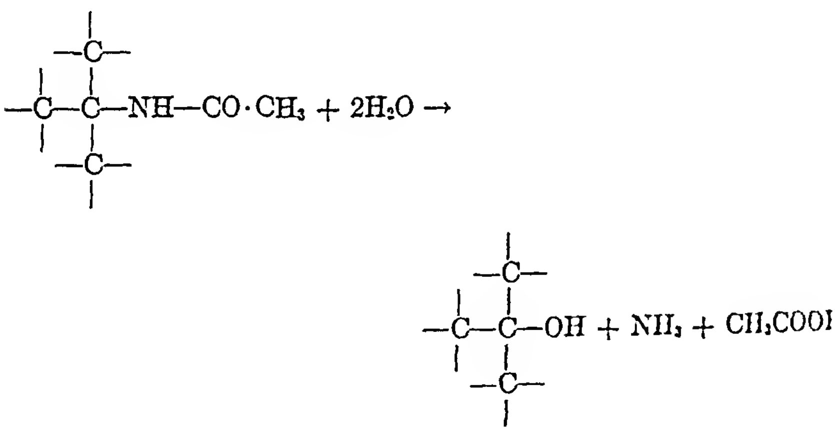
* Tests indicate the presence of acetic acid.

chitin. This is attributed to the vulnerability of the amino group to hydrolysis in the chitin molecule and to the fact that most of the chitinoclasts utilize ammonia nitrogen (table 1). Nearly all of the bacteria which attack chitin liberate an excess of ammonia therefrom. The data in table 2 show that the ammonia reaction becomes positive before there is any physical evidence that the chitin is being attacked by such bacteria; and some cultures never dissolve the chitin strips although ammonia is liberated in abundance.

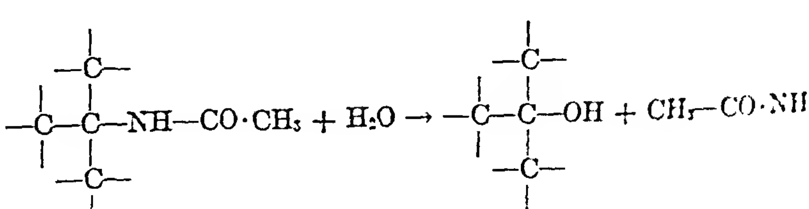
Some chitinoclasts produce enough acid from chitin to change

the reaction of the sea-water menstruum from an initial pH 7.6 to pH 5.0 as illustrated by the data in table 3. Also it will be noted that some cultures form acid before the chitin undergoes visible dissolution. All of the cultures tested which produced acid likewise liberated ammonia from chitin but there were many *cultures which liberated ammonia without changing the pH of* the chitin medium. Acetic acid has been identified as one of the products of the bacterial decomposition of chitin and the odor of some cultures resembles butyric acid. Folpmers (1921) has reported butyric acid formation by mixed cultures of chitinovors.

The production of acetic acid and ammonia suggests that the chitinoclasts attack the chitin molecule by hydrolyzing the acetylated amino groups:



The reaction probably takes place in two steps, with acetic acid first being liberated after which deaminization occurs. Or, it is possible that the carbon-nitrogen linkage is hydrolyzed first, thereby liberating acetamide:



Hydrolysis of the acetamide would give rise to acetic acid and ammonia. Some chitinoclasts may merely attack the acetylated amino side-chains leaving the essential carbon and oxygen linkages in the chitin molecule intact, which would account for the fact that some grow freely in chitin medium and even liberate acetic acid and ammonia in excess without dissolving the chitin. Incidentally, most of the marine chitinoclasts are able to derive their nitrogen and carbon or energy requirements from ammonia and acetic acid respectively (table 1). Of course, many of the chitinoclasts attack the essential carbon and oxygen linkages of the chitin molecule as manifested by the complete disintegration of the chitin strips and the liberation of reducing sugars. Reducing sugars were detected in four of the ten different cultures grown in chitin medium when tested with Benedict's reagent.

Several cases of obligate periphytism (ZoBell, 1937) have been observed among the chitinoclasts. For example, one culture covered the chitin strip with a heavy orange growth while the surrounding menstruum remained quite colorless. When a loopful of the liquid was transferred to another tube of chitin medium no growth developed. However, when a bit of the orange growth scraped from the original chitin strip was used as the inoculum, bacteria began to develop at once on the new chitin strip. This indicates that most of the bacteria were growing attached to the chitin, with few or none in the menstruum.

About one-fourth of the initial raw cultures were definitely pigmented and more produced various tinctorial changes, including fluorescence on the chitin strips. Yellow, orange, green fluorescence and light brown predominated in the order given. Some of the cultures produced an abundance of yellow or orange water-soluble pigment which diffused throughout the medium. Pigmentation has proved to be very ephemeral; so, until more is learned concerning the factors which influence pigment production by marine bacteria, this characteristic must be used with extreme caution for differentiating species.

Nearly all of the organisms included in this study are strict aerobes although a few of them are facultative anaerobes. This does not mean that most marine chitinoclasts are aerobic because

the isolation procedures were designed to obtain only aerobes. Marine anaerobes which digest chitin have been demonstrated in oval tube deeps (Rittenberg, *et al.*, 1937) containing a chitinous medium from which oxygen was excluded by a leuco-methylene blue agar seal. Indications are that there are many anaerobic chitinoclastic bacteria in the highly reduced bottom deposits of the sea.

Since over 80 per cent of the ocean floor is perpetually colder than 4°C., it is significant that chitin digestion by marine bacteria has been demonstrated at refrigeration temperatures (0 to 4°C.). ZoBell (1934) has shown that while few, if any, of the bacteria isolated from the sea are obligate psychrophiles, most of them are slowly, yet definitely, biochemically active at 0 to −2°C. and perhaps even lower temperatures. Johnson (1932) isolated chitinovors growing on crabs packed in ice.

SUMMARY

Chitinoclastic bacteria have been found to be quite widely distributed in marine sediments, animals and sea water off the coast of southern California.

Thirty-one different pure cultures isolated from marine materials have been studied.

Marine chitinoclasts are described which can derive their complete carbon or energy and nitrogen requirements from chitin. Others require supplementary carbon compounds but can utilize nitrogen from chitin. Still others attack chitin only in the presence of simple carbon and nitrogen sources.

Many chitinoclastic bacteria liberate ammonia or acid from chitin; processes which may or may not be accompanied by its dissolution. Reducing sugars have been detected in some cultures as a decomposition product.

REFERENCES

Benecke, W. 1905 Über *Bacillus chitinovorous*, einen Chitin-zersetzenden Spaltpilz. Bot. Ztg., Abt. 1, 63, 227–242.

Benton, Anne G. 1935 Chitinovorous bacteria. A preliminary survey. Jour. Bact., 29, 449–463.

Bertel, R. 1935 Les bactéries marine et leur influence sur la circulation de la matière dans la mer. Bull. Inst. Oceanograph., 672, 1–12.

Buchanan, R. E., and Fulmer, E. I. 1930 Physiology and Biochemistry of Bacteria. The Williams & Wilkins Company, 1, 98.

Bucherer, H. 1935 Über den mikrobiellen Chitinabban. Zentralbl. f. Bakt., Abt. II, 93, 12–24.

Diehl, J. M. 1936 Pflanzlichen Chitin. Chem. Weekblad., 33, 36–38.

Folpmers, T. 1921 Der Zersetzung des Chitins und des Spaltungsproduktes desselben, des Glucosamins, durch Bakterien. Chem. Weekbl., 18, 249–258.

Hess, E. 1937 Chitinovorous bacteria on live lobsters. Progress Reports, Biol. Board of Canada, No. 19, 10–11.

Iterson, G. van, Meyer, K. H., and Lotmar, W. 1936 Über den Feinbau des pflanzlichen Chitins. Rec. trav. chim., 55, 61–63.

Jensen, H. L. 1932 The microbiology of farmyard manure decomposition in soil. Jour. Agric. Sci., 22, 1–25.

Johnson, Delia E. 1932 Some observations on chitin-destroying bacteria. Jour. Bact., 24, 335–340.

Johnstone, J. 1908 Conditions of Life in the Sea. Cambridge Univ. Press, pp. 332.

Rammelberg, G. 1931 Beitrag zur Kenntnis des Chitins der Pilze und Krabben. Bot. Arch., 32, 1–37.

Rittenberg, S. C., Anderson, D. Q., and ZoBell, C. E. 1937 Studies on the enumeration of marine anaerobic bacteria. Proc. Soc. Exper. Biol. and Med., 36, 652–653.

Stuart, L. S. 1936 A note on halophilic chitinovorous bacteria. Jour. Amer. Leather Chem. Assoc., 31, 119–120.

Waksman, S. A., et al. 1933 Studies on the biology and chemistry of the Gulf of Maine. III. Bacteriologic investigations of the sea water and marine bottoms. Biol. Bull., 64, 183–205.

ZoBell, C. E. 1934 Microbiological activities at low temperatures with particular reference to marine bacteria. Quart. Rev. Biol., 9, 460–466.

ZoBell, C. E., and Anderson, D. Q. 1936 Vertical distribution of bacteria in marine sediments. Bull. Amer. Assoc. Petrol. Geol., 20, 258–269.

SPECIES SPECIFIC IMMUNITY TO HEMOLYTIC STREPTOCOCCUS INFECTIONS INDUCED IN WHITE MICE BY IMMUNIZATION WITH AN R VARIANT OF AN ERYSIPELAS STRAIN

The Significance of Cell Autolysis in Relation to Antibody Response

SOPHIE SPICER and EMILY L. BLOOM

Bureau of Laboratories, Department of Health, New York City

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The numerous attempts of different investigators to produce effective antistreptococcus sera and vaccines have thus far met with little success. The method followed by most workers in the attempt to produce an antibacterial serum for hemolytic streptococci is to use so-called type strains for the immunization of the animals. However, the types of hemolytic streptococci are too numerous, as well as too narrow in their specificity. Topley (1934) among others, points out the limitations imposed by specificity, stating:

> If the disease were caused by not more than two or three antigenic types, we might, by the injection of one of them, produce an appreciable reduction in the incidence of the disease as a whole. What, of course, we actually do, is to try to include in our vaccine all those types to which a person is likely to be exposed. If these number only two or three the problem is easy, but where the number of types is very large we can hardly hope to obtain the same results, partly because there is a limit to the number of different types that can be effectively combined in a vaccine, partly because the relative importance of different types may vary from place to place and from time to time.

If we accept the assumption that antibacterial protection in streptococcus infections runs parallel with the agglutinative

types, (Williams, 1932; Dochez, Avery and Lancefield, 1919) what chance is there for the invading organism in a given streptococcal infection to be identical with the strain or strains used in the preparation of the serum?

The urgent need for an antistreptococcus (antibacterial) serum and vaccine is obvious. Clinical experience has shown that the action of the antiscarlatinal serum in use today is antitoxic, but not antibacterial, as is seen from the ineffectiveness of such serum in the invasive stage, or on the complications of the disease. It is the practice of most laboratories in the production of antiscarlatinal serum to inject the animals with the cells, as well as the toxin of the strain used, in the hope that the serum will be antibacterial as well as antitoxic. Such serum would probably be effective if the invasive organism were identical with the strain used in the production of the serum, but as we have pointed out before, the chances are very remote.

It has been shown, (Parrish and O'Kell, 1928; O'Kell, 1932; Wheeler, 1932) that the toxins of strains from erysipelas, puerperal septicemia, and other streptococcal infections are neutralized by antiscarlatinal serum when tested on the skin. For this reason many workers advocate the use of antiscarlatinal serum in these conditions. But when we realize that these infections are more the result of the invasion by the organisms, than of their toxic effect, we can understand why the clinical use of the antitoxic serum in these diseases is so rarely, if ever, effective (Bertolini, 1921; White, 1927; Francis, 1928; McCann, 1928; Besson, 1929; Jacobsen, 1929). Obviously, what is needed for the production of an effective antistreptococcus serum is a strain of broad, antibacterial valence as well as broad antitoxic valence; one that would be species specific rather than type specific. We believe it not impossible to find a strain of hemolytic streptococcus which would be equivalent to Dochez NY 5 in its broad valence in the bacterial antibody response. We also believe that the solution to the problem may lie in a deeper knowledge of bacterial variation.

From a recent study (Spicer, 1936) of variation of hemolytic streptococci, we have found that in the majority of cases vaccines

made from R variant strains give better protection when tested on white mice than those prepared from the original or parent S strains. One rough variant, in particular, gives very high protection against heterologous, virulent strains of hemolytic streptococci from different disease conditions, such as scarlet fever, erysipelas, puerperal fever and general septicemias. It has evidently both a high antigenic potency and a wide protective valence. The protection afforded to the animals immunized with the vaccine made from this strain, is species specific. We therefore thought it worth while to test the effectiveness of this strain further, and also to modify the procedure in the preparation of the vaccines made from it.

The rough variant referred to was designated as B_3R. It was derived from B_3, an erysipelas strain which is the representative member of type II erysipelas (Williams, 1932). This rough variant was described in a previous publication (Spicer, 1933) in which the results of a study of variation of seven hemolytic streptococcus type strains were reported. For the sake of simplicity the strains studied were designated at that time as the types which they represented and the rough strains as the R variants of the homologous type strains. B_3R, therefore, was referred to as the rough variant of type II erysipelas. A brief description of the cultural and morphological characteristics of B_3R and its parent strain B_3 will follow. A detailed account of the origin, serological and other characteristics of this R variant will be found in previous communications (Spicer, 1933 and 1936).

B_3 gives the colony morphology supposedly typical of erysipelas strains, that is, a slightly granular, convex colony of regular contour and a good zone of hemolysis. In liquid media it grows with a cloudy supernatant and a granular deposit. The stained smear from liquid culture media presents short chains of small cocci, while those from solid media show small cocci in pairs, singly, or in very short chains of four to six elements. B_3R, on the other hand, is a typical R variant, as can be seen from the photographs in a previous publication (Spicer, 1933), and corresponds to the R phase of other bacterial species. It forms large, flat, extremely rough colonies, with irregular fringed bor-

ders, loops of chains of large cocci constituting the fringe. It gives a large zone of hemolysis. The stained specimens, both from liquid and solid culture media, present tangled masses of exceedingly long chains of large, encapsulated cocci. In liquid media this variant grows with a heavy sediment and a clear supernatant. On shaking, the sedimented growth rises in a cloud of large flaky masses which soon settle again to the bottom of the tube. Both B_3R and its parent strain B_3 are non-virulent for mice.

B_3R closely resembles the R variant type of hemolytic streptococcus recently described by Dawson (1933–4), in its cultural and morphological characteristics and also the new rough variant form of pneumococcus recently described by the same author (Dawson, 1932–3). It may perhaps be correlated with the R form of Loewenthal (cited by Ward and Lyons, 1935), especially by its avirulence for mice. It does not correspond to any of Ward and Lyons recently described four variants of hemolytic streptococcus which they designate as F, M, attenuated M and C.

Working with vaccine made from B_3R, we found that when prepared in the usual routine manner (formalinized) it was toxic for the mice; that is, it made them sick and even killed a small percentage. Those mice which survived, however, were very well protected against subsequent injections of highly virulent, unrelated strains of hemolytic streptococci. An attempt was therefore made to detoxify the vaccine. The method used, and the results of the protection tests with the treated vaccine as compared with those of the untreated vaccine, will be given subsequently.

METHODS

Preparation of vaccine. The general technique used for the preparation of the formalinized vaccine is as follows: The strain designated B_3R is first streaked on blood agar plates and examined for purity of type. It is then planted on vitamin agar slants in pint Blake bottles and incubated for eighteen hours. The growth from the Blake bottles is washed off with saline solution, the cells collected into centrifuge tubes and centrifugated

for forty-five minutes. The cells are washed once and resuspended in formalinized saline solution (0.1 per cent formalin in 0.85 per cent sodium chloride solution). The vaccine is standardized by comparing the turbidity of the cell suspension with a set of Fuller's earth standard turbidity tubes and is equivalent to about three billion organisms per cubic centimeter.

Method of detoxication. It was found that treating the vaccine with alcohol, and ether, not only renders it non-toxic, but gives better protection to animals in the majority of cases. The procedure of treatment is as follows: The cells are grown and collected as described above, washed once with absolute alcohol, resuspended in absolute alcohol, kept in a water bath at 50°C. for thirty minutes and stirred occasionally. After centrifugating for ten minutes and decanting the alcohol, ether is added in amount of ten times the bulk of the cells, mixed with a glass rod for thirty minutes and centrifugated for two minutes at low speed with centrifuge cover open. The ether is then decanted, the tubes plugged loosely and placed in the icebox. At the end of eighteen hours the cells are perfectly dry. They are suspended in formalinized saline, standardized, and kept in the icebox until used. The vaccine so prepared is designated as B_3RA.

Protection tests. White mice, ranging from 18 to 21 grams in weight, were used in the protection tests. The mice received three injections intraperitoneally on alternate days, first and second injections consisting of 0.5 cc. and the third injection of 1 cc. of vaccine. On the seventh day after the last injection the mice were tested for immunity with virulent, heterologous S cultures. They could not be tested against the homologous S strain (B_3) as this was entirely non-virulent and its virulence could not be raised by mouse passage. All the test strains used in the protection tests were rendered virulent by mouse passage so that 10^{-7} to 10^{-8} cc. killed a mouse in from twenty-four to forty-eight hours. The cultures used in the tests were transplanted into serum broth (10 per cent horse serum), grown for six hours, transplanted into plain broth, incubated for eighteen hours and used for dilutions. The dilutions were made with broth after the turbidity of the cultures was brought to a stand-

TABLE 1

Protection test—Mice vaccinated with B_1RA vaccine, tested against S-215, M. 51

MOUSE NUMBER	DATE AND AMOUNT OF VACCINE			TEST CULTURE 6/13	RESULTS	AUTOPSY FINDINGS	PERCENTAGE PROTECTION
	6/2	6/4	6/6				
	cc.	cc.	cc.				
1	0.5	0.5	1	10^{-3}	Died 48 hours	Hem. strep.	100,000 M.L.D. 35%
2	0.5	0.5	1	10^{-3}	Died 72 hours	Hem. strep.	
3	0.5	0.5	1	10^{-3}	Died 72 hours	Hem. strep.	
4	0.5	0.5	1	10^{-3}	Died 5 days	Hem. strep.	
5	0.5	0.5	1	10^{-3}	Saved		
6	0.5	0.5	1	10^{-4}	Died 4 days	Hem. strep.	10,000 M.L.D. 90%
7	0.5	0.5	1	10^{-4}	Saved		
8	0.5	0.5	1	10^{-4}	Saved		
9	0.5	0.5	1	10^{-4}	Saved		
10	0.5	0.5	1	10^{-4}	Saved		
11	0.5	0.5	1	10^{-5}	Died 72 hours	Hem. strep.	1,000 M.L.D 80%
12	0.5	0.5	1	10^{-5}	Saved		
13	0.5	0.5	1	10^{-5}	Saved		
14	0.5	0.5	1	10^{-5}	Saved		
15	0.5	0.5	1	10^{-5}	Saved		
16	0.5	0.5	1	10^{-6}	Saved		100 M.L.D. 100%
17	0.5	0.5	1	10^{-6}	Saved		
18	0.5	0.5	1	10^{-6}	Saved		
19	0.5	0.5	1	10^{-6}	Saved		
20	0.5	0.5	1	10^{-6}	Saved		
21	0.5	0.5	1	10^{-7}	Saved		10 M.L.D. 100%
22	0.5	0.5	1	10^{-7}	Saved		
23	0.5	0.5	1	10^{-7}	Saved		
24	0.5	0.5	1	10^{-7}	Saved		
25	0.5	0.5	1	10^{-7}	Saved		
26	0.5	0.5	1	10^{-8}	Saved		1 M.L.D. 100%
27	0.5	0.5	1	10^{-8}	Saved		
28	0.5	0.5	1	10^{-8}	Saved		
29	0.5	0.5	1	10^{-8}	Saved		
30	0.5	0.5	1	10^{-8}	Saved		

TABLE 1—*Concluded*

Controls S-215, M. 51

MOUSE NUMBER	TEST CULTURE 6/13	RESULTS	AUTOPSY FINDINGS	COLONY COUNT	TURBIDITY STANDARD
1	10^{-6}	Died 24 hours	Hem. strep.	412	
2	10^{-6}	Died 24 hours			
3	10^{-6}	Died 24 hours			No. 350
4	10^{-6}	Died 24 hours			
5	10^{-6}	Died 24 hours			
6	10^{-7}	Died 24 hours	Hem. strep.	30	
7	10^{-7}	Died 24 hours			
8	10^{-7}	Died 48 hours			
9	10^{-7}	Died 48 hours			
10	10^{-7}	Died 48 hours			
11	10^{-8}	Died 48 hours	Hem. strep.	3	
12	10^{-8}	Died 48 hours			
13	10^{-8}	Died 48 hours			10^{-8} M.L.D.
14	10^{-8}	Survived			
15	10^{-8}	Survived			
16	10^{-9}	Survived		0	
17	10^{-9}	Survived			
18	10^{-9}	Survived			
19	10^{-9}	Survived			
20	10^{-9}	Survived			

ard. Blood-agar pour plates for colony count were made from the various dilutions as an added check on the size of the dose. Series of thirty mice were used for each test, five for each dilution of culture. Twenty normal (non-vaccinated) mice were injected with the test culture for controls. Tests were repeated when results were not clear-cut or when control mice did not die regularly. Animals surviving six days were considered to be protected. Table 1 is a protocol of one of the protection tests.

Results of the protection tests. As seen from table 2, there was a considerable rise in the degree of protection when the animals were immunized with the detoxified vaccine. In every case in which the mice received the treated vaccine they appeared well during the period of vaccination and no animals were lost.

TABLE 2

Comparative protection tests on white mice immunized with B_3R and B_3RA vaccines against virulent heterologous strains

VACCINE	TEST STRAIN	SOURCE	PERCENTAGE PROTECTION IN NUMBER OF M.L.D.'s					
			1	10	100	1,000	10,000	100,000
B_3R	28_1	Throat, scarlet fever	100	100	80	40	15	–
B_3RA	28_1		80	100	80	75	20	15
B_3R	E-111	Skin, post-scarlet fever erysipelas	100	100	100	60	40	–
B_3RA	E-111		100	100	80	60	50	–
B_3R	E-109	Skin, erysipelas	80	80	70	20	0	–
B_3RA	E-109		100	100	30	40	40	–
B_3R	E-104	Skin, erysipelas	100	100	60	20	20	–
B_3RA	E-104		70	40	0	30	0	–
B_3R	E-106	Skin, erysipelas	100	95	75	25	–	–
B_3RA	E-106		80	80	40	30	0	–
B_3R	P-301	Blood, puerperal fever	100	100	90	65	65	–
B_3RA	P-301		100	75	40	40	40	–
B_3R	P-302	Blood, puerperal fever	100	100	95	20	25	–
B_3RA	P-302		100	100	100	30	–	–
B_3R	S-208	Blood, septicemia	60	55	80	40	–	–
B_3RA	S-208		80	80	80	35	70	55
B_3R	S-206	Blood, septicemia	100	95	90	80	–	–
B_3RA	S-206		100	100	100	100	100	65
B_3R	S-211	Blood, septicemia	60	20	20	0	0	–
B_3RA	S-211		70	40	40	50	0	–
B_3R	S-215	Blood, septicemia	80	100	80	100	45	–
B_3RA	S-215		100	100	100	80	90	35
B_3R	S-218	Blood, pneumonia	80	40	60	0	0	–
B_3RA	S-218		100	100	100	50	75	–

0 denotes no protection; – denotes not tested.

Percentages were arrived at as follows: Surviving six days, 100 per cent protection; surviving five days, 75 per cent protection; surviving four days, 50 per cent protection; dying within three days, no protection.

The problem was also attacked from a different angle. We know that hemolytic streptococci autolyze with difficulty, and in culture media to a very slight degree, if at all. There are many accounts in the literature of attempts to bring about the autolysis of these organisms (Kantorovicz, 1909; Bürgers, Schermann and Schreiber, 1911–12; Zinsser and Mueller, 1928). It is only by extreme physical means such as alternate freezing and thawing, or by treating with very strong chemicals such as 10 per cent antiformin, that this can be accomplished, and then only to a limited extent. This leads us to believe that the antibody response to this organism may be against the antigenic substance on the cell surface but not against the antigenic constituents of the inner cell body.

Topley (1934) describing the mechanism of antitoxic and antibacterial immunity says: "In the case of a toxin the result of the antigen-antibody union is neutralization. . . . In the case of bacteria, or virus particles, the antigen-antibody union occurs on the surface of the parasite and results in the deposition thereon of scattered or continuous patches of antibody globulin." Northrop and De Kruif (1921–2 and 1922–3),. Shibley (1926), Eagles (1930) and others showed that globulin from immune serum is deposited on the bacterial cell during specific bacterial agglutination. Jones and Little (1933), on the basis of this knowledge, performed a series of experiments by which they showed that bacteria increase in volume when treated with specific agglutinins, giving actual measurements to prove their point.

The antibodies produced by hemolytic streptococci (besides the antiexotoxins of course) are thus evidently active against these antigenic constituents on the bacterial surface. It is interesting at this point to mention the effect of washing hemolytic streptococcus antigens used in agglutination. We (Spicer, 1930) have found a gradual reduction in the titer of the agglutinating serum from 12,800 with the unwashed to 800 with the antigen washed three times. This could not have been the result of absence of electrolyte, since the antigens had been washed and suspended in saline solution.

A bacterium that does not autolyze in the normal body fluids

would thus stimulate the production of antibodies against the soluble surface antigenic fractions but not against the antigenic content of the inner cell body. With this in mind, experiments were undertaken to effect the disruption of the organisms by physical means, so as to free the somatic antigenic fractions. A vaccine thus prepared should theoretically produce antiendotoxins in addition to the other antibodies. Alternate freezing

TABLE 3

Comparative protection tests on white mice with B_3RA vaccine and B_3RA vaccine, frozen and thawed thirty-five times, against erysipelas, post-scarlet fever erysipelas, puerperal fever, meningitis and general septicemia strains

VACCINE	FROZEN AND THAWED	TEST STRAIN	SOURCE	PERCENTAGE PROTECTION IN NUMBER OF M.L.D.'S					
				1	10	100	1,000	10,000	100,000
B_3RA	–	E-104	Skin, erysipelas	70	40	20	30	0	–
B_3RA	35×	E-104		100	70	100	60	100	70
B_3RA	–	E-111	Skin, post-scarlet fever erysipelas	100	100	80	60	50	–
B_3RA	35×	E-111		100	100	70	100	80	60
B_3RA	–	P-301	Blood, puerperal fever	100	75	40	40	40	–
B_3RA	35×	P-301		100	100	100	100	95	–
B_3RA	–	M-139	Spinal fluid, meningitis	80	100	80	80	40	20
B_3RA	35×	M-139		100	100	100	100	60	40
B_3RA	–	S-211	General septicemia	70	40	40	50	0	–
B_3RA	35×	S-211		100	100	100	100	40	–
B_3RA	–	S-218	Blood, pneumonia	100	100	100	50	75	–
B_3RA	35×	S-218		100	100	100	80	80	–

× denotes times.

and thawing was chosen for this purpose. The results of the protection tests in the mice immunized with this vaccine are given above.

We found that alternate freezing and thawing when applied from eight to ten times did not alter the degree of protection by the vaccine. There was a definite rise in the degree of protection after freezing and thawing twenty to twenty-five times. The

maximum effect was achieved by freezing and thawing about thirty-five times. Further treatment of the vaccine by this process reduced the degree of animal protection. Table 3 shows the comparative results of the protection tests on white mice with this vaccine.

DISCUSSION

Hadley (1927), treating the subject of Dissociation and Immunologic Response, states: "The artificial production of cyclogenic variants, and a careful comparison of the relative immunizing power of these dissociates, is at present one of the most important fields of investigation in vaccine therapy." Although an enormous amount of work on bacterial variation has since been performed by various workers, comparatively little has been done on that phase of the phenomenon. De Kruif showed that a solid immunity can be produced in rabbits by a single, small injection of R culture of *Bacterium lepisepticum* against the virulent homologous S type. Cowan (1923) working with a hemolytic streptococcus also showed that definite resistance can be produced in white mice vaccinated with the R type of that organism. Maltaner (1934), more recently, vaccinated rabbits with rough and smooth variants of typhoid bacillus and found that the rough vaccines gave distinctly better protection than the smooth. In a recent paper (Spicer, 1936) we also showed that vaccines made from R strains of hemolytic streptococci give better protection to white mice than those prepared from the parent S strains.

Tillet (1928) working with pneumococci found that R variants are species specific in their immunological response. Cowan (1923) showed that the immunity produced in mice with R types of hemolytic streptococci was not confined to the S type of the homologous strain, but also protected the animals against S types of heterologous strains.

As can be seen from the results of the protection tests recorded above, the immunity induced in the animals with our variant B_3R is also species specific. Itself an erysipelas strain, it protects mice against highly virulent strains from erysipelas, scarlet fever,

puerperal sepsis, spinal meningitis and general septicemias. Contrary to the popular belief that virulent strains protect better than avirulent, B_3R, although avirulent, gives high protection against virulent S strains. Another interesting point relative to this strain is its toxic effect when introduced as a vaccine into the peritoneal cavity of the mouse, and the ease with which it can be rendered atoxic.

It has been shown (Bordet, 1897; Gay and Morrison, 1923; Gay and Clark, 1929; Kolmer, 1923) that the immune response in streptococcal infections is phagocytic. Therefore the antibodies influencing the reaction must be those that induce phagocytosis, i.e. opsonins and agglutinins. These, as Topley indicated, react with the surface antigenic fractions and are not antiendotoxic. When we introduce a vaccine made from whole, killed cells into the body of an animal the antibody response may therefore be against the ectoplasmic substance and not against any of the antigenic elements, of which the cell cytoplasm or the somatic portion of the cell consists. My reason for this belief is based on the fact that hemolytic streptococci autolyze with difficulty, if at all, *in vitro*, and probably with as much difficulty in the body of the normal animal. The antibody response, therefore, would be against the intact organism and not against the endotoxins. Phagocytosis is not always followed by destruction of ingested bacteria. When the ingested bacteria are not destroyed by the cells the phagocytosis may even aid in the dissemination or the maintenance of the infection. It was shown (Topley and Wilson, 1929) that bacteria may remain alive for considerable periods within the sessile macrophages. Experiments carried out by Roux and Jones (1916) suggest that ingestion by living cells may even protect bacteria from the lethal action of bactericidal sera. Our contention, therefore, is that phagocytic response alone in any infection that is of an invasive nature is not sufficient. Endotoxin neutralizing immune bodies, in addition to the phagocytic, are essential. In order that these organisms may stimulate the production of antiendotoxins, the cells would have to be disrupted and the endotoxins freed from the apparently tough outer membrane of the cell body, thus releasing the potential

antigenic activity of the true somata. We chose the physical process of alternate freezing and thawing to avoid any alteration in the antigenic structure of the cells which chemical reagents are apt to cause. However, no visible change could be noted in the cells of the frozen and thawed vaccine when examined microscopically on the slide, except for the loss of ability of some of the cells to take the Gram stain. They all appeared intact. We have no explanation for the higher protection obtained with the frozen and thawed vaccine, except that the process may have so altered the cell membrane as to effect dissolution in the animal body fluids more easily. Other methods of cell dissolution may prove still more effective.

SUMMARY AND CONCLUSIONS

A rough variant of an erysipelas strain, used as vaccine, protected white mice against virulent, unrelated strains from different pathological conditions caused by hemolytic streptococci.

Although the variant and the original S strain from which the variant was derived are avirulent, the vaccine made from the R variant was toxic for the mice, making the animals sick during vaccination and even killing a small percentage.

Those animals that survived were very well protected against subsequent injections with virulent, heterologous strains of hemolytic streptococci from diverse pathological conditions.

Treating the vaccine with alcohol and ether rendered it atoxic for the animals.

The protection afforded by the detoxified vaccine was higher in most cases than with the untreated vaccine.

Vaccines subjected to repeated freezing and thawing gave still higher protection to the mice.

The significance of cell autolysis in immunological reactions is discussed.

Results of experiments conducted seem to confirm the theoretical considerations brought forward.

REFERENCES

Bertolini, G. 1921 Über die Immunitätsreaktionen im Wochenbettfieber, mit Berücksichtung der üblichen Therapie. Centralbl. f. Bakteriol., etc., 86, I Abt., Orig., 266.

Besson, A. 1929 Cited by Pickett-Thomson. Pathogenic Streptococci, 5 Ann. Pickett-Thomson Res. Lab. The Williams & Wilkins Company, Baltimore.

Bordet, J. 1897 Infection streptococcique. Ann. Inst. Pasteur, 11, 177.

Bürgers, Schermann, und Schreiber 1911–1912 Über die Auflösungserscheinungen von Bakterien. Zeitschr. f. Hygiene u. Infektionskrankheiten, 70, 119.

Cowan, M. L. 1923 Variation phenomena in streptococci. Further studies on virulence and immunity. Brit. Jour. Exp. Path., 4, 241.

Dawson, M. H. 1932–33 Dissociation of pneumococcus: a new colony variant Proc. Soc. Exp. Biol. and Med., 30, 806.

Dawson, M. H. 1933–34 Bacterial variation in pneumococcus and streptococcus hemolyticus. Proc. Soc. Exp. Biol. and Med., 31, 590.

Dochez, A. R., Avery, O. T., and Lancefield, R. C. 1919 Studies on the biology of the streptococcus. Jour. Exp. Med., 30, 179.

Eagles, H. 1930 Specific agglutination and precipitation. I. Mechanism of the reaction. Jour. Immunol., 18, 393.

Francis, T. 1928 Studies on pathogenesis and recovery in erysipelas. Jour. Clin. Invest., 6, 221.

Gay, F. P., and Clark, A. R. 1929 Further note on the relative protection of polymorphonuclear and mononuclear cells in local streptococcus infection. Proc. Soc. Exp. Biol. and Med., 27, 995.

Gay, F. P., and Morrison, L. E. 1923 Clasmatocytes and resistance to streptococcus infection. Studies in streptococcus infection and immunity. Jour. Inf. Dis., 33, 338.

Hadley, P. 1927 Microbic dissociation. Jour. Inf. Dis., 40, 1.

Jacobsen, A. W. 1929 Erysipelas: report of a case. Jour. Amer. Med. Assoc., 93, 374.

Jones, F. S., and Little, R. B. 1933 Changes in bacterial volume as the result of specific agglutination. Jour. Exp. Med., 57, 721, 729.

Kantorovicz, A. 1909 Bakterien-antifermente und Bakteriolyse. Münch. med. Wochenschr., 56, 1.

Kolmer, J. A. 1923 Infection, Immunity, and Biologic Therapy. W. B. Saunders Co., Philadelphia, Pa.

Maltaner, F. 1934 A study of "smooth" and "rough" forms of the typhoid bacillus in relation to prophylactic vaccination and immunity in typhoid fever. Jour. Immunol., 26, 161.

McCann, W. S. 1928 The serum treatment of erysipelas. Jour. Amer. Med. Assoc., 91, 78.

Northrop, J. H., and DeKruif, P. H. 1921–22 The stability of bacterial suspensions. Jour. Gen. Physiol., 4, 639, 655; 1922-23, 5, 139, 605.

O'Kell, C. C. 1932 Hemolytic streptococci in infective disease. Lancet, Jan.–June, 761, 815, 867.

Parish, H. J. and O'Kell, C. C. 1928 Two studies of streptococcal infections. I. Hemolytic streptococcal infections in the rabbit. Lancet, Jan.–June, 746.

Roux, P., and Jones, F. S. 1916 The protection of pathogenic microörganisms by living tissue cells. Jour. Exp. Med., 23, 601.

SHIPLEY, G. S. 1926 On the mechanism of the agglutination of bacteria by specific agglutinating serum. Jour. Exp. Med., 44, 667.

SPICER, S. 1930 A quick method for preparing homogeneous suspensions of hemolytic streptococcus antigens for agglutinin testing. Jour. Immunol., 19, 445.

SPICER, S. 1933 A study of variation in hemolytic streptococci from scarlet fever and erysipelas. Jour. Bact., 26, 505.

SPICER, S. 1936 A study of variation in hemolytic streptococci from scarlet fever and erysipelas. Jour. Bact., 32, 105.

TILLET, W. S. 1928 Active and passive immunity to pneumococcus infection induced in rabbits by immunization with 'R' pneumococci. Jour. Exp. Med., 48, 791.

TOPLEY, W. W. C. 1934 Principles of immunization and preventive disease. Lancet, June 2, 1185.

TOPLEY, W. W. C., AND WILSON, G. S. 1929 The Principles of Bacteriology and Immunity. William Wood & Co., New York.

WARD, H. K., AND LYONS, C. 1935 Studies on the hemolytic streptococcus of human origin. I. Observations on the virulent, attenuated, and avirulent variants. Jour. Exp. Med., 61, 515.

WHEELER, M. W. 1932 Notes on antigenic activity of hemolytic streptococci from different types of infections. Jour. Immunol., 24, 311.

WHITE, E. R. 1927 Puerperal and abortion sepsis. Med. Jour. Australia, 2, 38.

WILLIAMS, A. W. 1932 Streptococci in Relation to Man in Health and Disease. The Williams & Wilkins Company.

ZINSSER, H., AND MUELLER, J. H. 1928 Antigenic properties of the bacterial cell and antibody reaction. The Newer Knowledge of Bacteriology and Immunology. E. O. Jordan and I. S. Falk, Editors. University of Chicago Press.

REDUCTION AND CONCENTRATION OF METHYLENE BLUE BY CERTAIN PATHOGENIC FUNGI[1]

JOHN W. WILLIAMS

Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Massachusetts

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Ability to concentrate and reduce methylene blue is of importance in differentiation of certain pathogenic fungi. To study concentration, a medium producing a predominantly subsurface growth (Williams, 1936, b and c; 1937, a and b) (0.25 per cent d-glutamic acid, Hoffman-LaRoche, 4 per cent glucose Difco, 1.5 per cent agar Difco, pH 5.6) is preferable. To study reduction, either the above medium or a medium producing a predominantly surface growth (Sabouraud's medium Difco) may be used. A 0.001 per cent concentration of the dye (methylene blue, U. S. P. medicinal, CI 922, National Aniline & Chemical Co.) is ideal.

Surface plants on 4 to 5 cm. straights of the above mediums were made and observed after 5, 7, 10, 15 and 30 days growth in diffused light at room temperature with tubes secured upright. Three or more plants of each organism were made, since, otherwise, failure of an organism to cover the surface or cracks in the medium rendered results less accurate. All reduced areas were checked by stabs whereupon color reappeared. Natural organismal pigment was discounted in tabulation of results.

RESULTS

Table 1 gives salient variations. Space does not permit description of all colors or shades noted. When numbers such as 5 to 15 occur they indicate day of beginning reduction and re-

[1] Contribution No. 111 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

TABLE 1

ORGANISM	SABOURAUD'S MEDIUM	GLUTAMIC ACID MEDIUM
Achorion schoenleinii	5–15; 7, ba; 10, la; 15, toba; 30, dga	10–15; 10, 5 mm. vm; 15, 1.5 cm. dc; 30, 2 mm. tdc (2×) 0.5 mm. dl
Candida candida	15–30; 15, tb–gth; 30, dc edge and raised areas	10, v–gth
Endodermophyton indicum	7–15; 15, edge dc, center g; 30, 1 mm. dc	15–30; 10, v–gth; 15, 2 mm. 0, dc; 30, 2 mm. dc (2.5×), ½ mm. dl
Endodermophyton tropicale	7–15; 15, bga; 30, dbga	15–30; 10, dc–gwth; 15, dcm; 30, 2 mm. t–dc (1.5×), 0.5 mm. dl
Endomyces capsulatus	7–15; 10, 1–gth; 30, ½ mm. tdc (1×)	10, v–gth; 30, 2 cm. dc (1×), rest lighter blue; lamellation
Endomyces dermatitidis	7–15; 10, 1 mm. 1 c; 15, tba, $\frac{1}{16}$ mm. tdl	15–30; 10, 1–gth; 30, 6 mm. dc (2.5×), 5 mm. tbg
Glenospora gammeli	7–30; 7, ba; 10, la; 15, bva; 30, ½ mm. b esp. edge	ic 15–30; 10, 1–gth; 15, 1.5 cm. dc; 30, 1 dc (1.5×), tdl to colorless
Geotrichum bachmann	5–15; 7, b; 7 to 30 b edge–gth	7–10; 7, $\frac{1}{16}$ mm. dl; 10, 3 mm. dc; 15, 3.5 dc; 30, 3 mm. dc (2×), ½ mm. dl if
Lichthemia sp. (saprophyte)	10–30; 10, ba; 15, tba; 30, 1 mm. b, 1.5 mm. tbg	15–30; 10, 2 cm. vc; 15, 1.5 cm. dc, dl; 30, 1.5 cm. dc (3×), white line; f
Microsporon audouini	7–30; 10, ba; 15, ga; 30, obga	10–30; 10, dc–gth; 15, 2 mm. tdc, r, +bg; 30, 2 mm. tb, 1 mm. br
Microsporon felineum	5–15; 7, ba; 10, va; 15, gba; 30, bga	10–30; 10, 3 mm. vb–gth; 15, 3 mm. dc; 30, 2 mm. dc.
Microsporon gypseum	5–15; 7, ba; 10, ga; 15, ga; 30, ga	7–30; 10, 3 mm. dc; 15, 2 mm. dc; 30, 2 mm. dc (1×), 0.25 mm. bl–dl–bl
Monosporium apiospermum	5–30; 10, tba; 15, tba; 30, bgl–edge	15–30; 10, 2 mm. vb–gth; 15, 0.5 cm. gb–dc; 30, 3.5 cm. dc (2.5×)
Monilia albicans	7–30; 30, tb–gth–esp. edge	10, v–gth
Oospora humi	5–15; 10, b–edge–gth; 15, tb–edge–gth; 30, ½ mm. dl	10–15; 10, 2 mm. v; 15, 2 mm. bc; 30, 2 mm. bc (2.5×); f
Scopulariopsis brevicaulis (saprophyte)	7–30; 15, tga; 30, tga; f	10–15; 10, 3 mm. dc; 15, 3 mm. tdc; 30, 3 mm. dbc; 3 mm. bl; f

TABLE 1—*Concluded*

ORGANISM	SABOURAUD'S MEDIUM	GLUTAMIC ACID MEDIUM
Trichophyton crateriforme	7–30; 7, ba; 10, va; 15, tb–gth, ga, 0.5 cm. dc; 30, doga	15–30; 10, vc–gth; 15, 0.5 cm. dc; 30, 2 mm. dc (3×), 0.5 mm. dl
Trichophyton decalvans	7–30; 10, 0.5 mm. dc; 15, ba; 30, 2 mm. bl–l	30, 1.5 cm. bc (1×) bl, reduction
Trichophyton gypseum	5–10; 5, 1 mm. dl; 10, vga; 15, ba; 30, 2 mm. b–bl	15–30; 10, vc–gth; 15, 3 mm. dc; 30, 3 mm. dc (2.5×), $\frac{1}{8}$ mm. bl–dl
Trichophyton gypseum-asteroides	5–30; 15, gra; 30, r–g–bl–s–a	5–15; 10, dc; 15, 2 mm. dc; 30, 1.5 mm. tbc, 1 mm. b–bl
Trichophyton gypseum lacticolor	5–30; 7, ba; 10, gba; 15, rga; 30, rga	10–15; 10, 3 mm. vc.–gth; 15, 2.5 mm. dc, dl; 30, 1 mm. tbc (2×) dl
Trichophyton interdigitale	5–15; 7, ba; 10, ba; 15, oga; 30, g–bl–ba	7–15; 10, 2 mm. b–gth; 15, 2 mm. dc, tdl; 30, 2 mm. dc very deep
Trichophyton louisianicum	5–30; 7, r–ba; 10, r–ga; 30, 1 mm. bg, 3 mm. tr	5–30; 10, 3 mm. gb; 15, r–bdl; 30, 1.5 tb, 1 mm. dc (1.5×), g, r; f
Trichophyton niveum	5–10; 5, 1 mm. dl; 7, ba; 10, ba; 15, bga; 30, 1 mm. bc	10–15; 10, 3 mm. dc; 15, 2 mm. dc, dl; 30, 2 mm. tb, $\frac{1}{8}$ mm. dl. deep color

duction to the bottom of the tube; when ic precedes them, reduction is incomplete. The following captions indicate: a, base of growth; b, blue (see bl); c, concentration; d, dirty (see dc and dl); f, diffusion; g, green (see gth); l, lavender (see dl and bl); m, mycelium; o, olive; r, brown (see nr); s, salmon; t, light; v, violet; bl, black; dc, dye concentration; dl, dye line; gth, growth; nr, no reduction; (2x) the approximate number of times the concentration is deeper than control mediums (this is given only when it can be approximated; too great concentration would prevent this).

DISCUSSION

Dye concentration occurs only in the upper 2 cm. of the straight in proximity to, and in, certain growths (area of increased metab-

olism). This correlates with localization of basic dyes injected into animals in areas of inflammation (Burrows, 1932). The dye line occurs at the base of the dye concentration or independently. Pronounced concentration and dye line occur in a similar situation in cysteine medium after autoclaving but when other amino-acids are substituted, only after growth of certain organisms. Successive dye lines similar to Liesegang rings occasionally occur. Subsurface growths do not occur at a depth greater than 2 cm.

The green frequently noted may be a combination of changed dye and yellow resultant from alkaline change in the mediums. The lavender, violet or purple occur at a definite time (10th day) and therefore seem biologically fundamental in time. Basically the various color changes seem dependent on combinations of oxidations and reductions of the various constituents.

Reduction time and its completeness varied with the organism. Twenty plants on W medium (4 per cent peptone, 1 per cent glucose, 1.5 per cent agar, pH 5.6) indicated that this ratio of peptone to glucose speeded reduction, possibly because of better buffering power of the medium (Williams, 1935 and 1936a).

It is evident from the table that a classification might be suggested. This is inadvisable because of limited number of strains.

SUMMARY

Methods and mediums for demonstrating concentration and reduction of methylene blue are discussed.

Importance of oxidation and reduction in color changes is emphasized. These changes are orderly, probably interpreting metabolic behavior of the organism. Their similarity in time suggests a fundamental biological factor dependent on time.

Marked dye concentration and dye line occur on glutamic acid medium only after organismal growth while on cysteine medium these occur after autoclaving, independent of organismal growth. Related oxidation-reduction changes are probably the interpretation in both instances.

REFERENCES

BURROWS, H. 1932 Some factors in the localization of disease. Covent Garden, W. C. Great Britain. Ballière, Tindall and Cox.

WILLIAMS, J. W. 1935 I. Effect of variation of ratios of dextrose to peptone on colonies of certain pathogenic fungi. Arch. Dermat. and Syph., 32, 893–914.

WILLIAMS, J. W. 1936a II. Effect of variation of ratios of dextrose to peptone on colonies of certain pathogenic fungi. Arch. Dermat. and Syph., 34, 15–34.

WILLIAMS, J. W. 1936b Invasiveness of skin infections caused by pathogenic fungi and subsurface mycelium. Science, 83, 396–397.

WILLIAMS, J. W. 1936c The possible importance of mediums causing surface and subsurface growth of pathogenic fungi to the diagnosis and treatment of disease. Lab. and Clin. Med., 22, 268–269.

WILLIAMS, J. W. 1937a Allergy, immunity and growth of pathogenic fungi in vivo and in vitro. Urol. and Cutan. Rev., 41, 117–128, 198–204.

WILLIAMS, J. W. 1937b Subsurface growth of pathogenic fungi on peptone, hair, pig skin and cysteine-cystine mediums. Arch. Dermat. and Syph., 36, 581–598.

THE DIFFERENTIATION OF PATHOGENIC STAPHYLOCOCCI FROM NON-PATHOGENIC TYPES[1]

GEORGE H. CHAPMAN, CONRAD BERENS, EDITH L. NILSON AND LILLIAN G. CURCIO

Clinical Research Laboratory (604 Fifth Avenue), and Laboratory of the Lighthouse Eye Clinic of the New York Association for the Blind, New York, N. Y.

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Although numerous *in vitro* tests have been proposed for differentiating staphylococci on the basis of pathogenicity the subject is confusing to many workers. This paper describes attempts to determine the significance of certain tests.

The following seem to have been the more common sources of confusion: (1) Most investigators have used a series of cultures which was too small for statistical analysis; (2) groups of cultures from different sources, or obtained at different times from the same type of source, may not yield the same proportion of positive results; (3) results based on the source of the strains usually ignore the possibility of error; (4) differences in technic, or in the interpretation of results, may be sufficient to cause two investigators to arrive at opposite conclusions; only infrequently has an author described tests, or their interpretation, with sufficient precision to permit others to duplicate them; (5) the dissociation of a strain, resulting in loss of one or more properties in some cells of the culture, causes it to become a mixture of variants with different pathogenic properties; and (6) pathogenic cultures degenerate or dissociate at different rates and in different ways, depending upon a number of poorly understood factors.

CULTURES STUDIED

Cultures were obtained through the courtesy of:

Dr. John E. Blair. Hospital for Joint Diseases. New York, N. Y.

[1] Aided by grants from the Ophthalmological Foundation, Inc.

Dr. Louis Gross. Louis Gross Diagnostic Laboratory. New York, N. Y.
Dr. Wayne N. Plastridge. Storrs Agricultural Experiment Station. Storrs, Conn.
Mr. George J. Kupchik. Bacteriology Department. Medical School. New York University.
Dr. Howard J. Shaughnessy. Illinois Department of Public Health.
Dr. Raymond V. Stone. Los Angeles County Health Department.
Dr. Luther Thompson. The Mayo Clinic.

Other cultures were isolated in our laboratories.

METHODS USED

Cultures were diluted to insure that discrete colonies would be obtained, spread on plates of oxalated rabbit-blood agar and incubated overnight. Variants with different hemolytic properties were separated. Sometimes a colony, which appeared to be non-hemolytic, proved to be hemolytic on subculture. In these instances the results were recorded as ⊕. The same symbol was used when a reaction was positive one time and negative another. When the results were intermediate or inconclusive the symbol ± was used.

The following tests, selected because they were considered most suitable for the majority of bacteriologists, were applied to the purified variants:

Pigment production. Determined by cultivating for 48 hours, preferably on Loeffler's medium, and examining a loopful of the culture. Because there is no sharp distinction between pigmented and non-pigmented strains this test sometimes is difficult to interpret.

Hemolysis. Determined by streaking a loopful of the culture across the surface of a plate of oxalated rabbit-blood agar and incubating overnight. Growths showing moderate or marked hemolysis were considered positive. Lesser degrees of hemolysis were considered negative.

Coagulase. Determined by mixing a loopful of the culture from solid medium with 0.5 cc. of fresh, oxalated human plasma

(citrated or heparinized plasma gave similar results). After shaking thoroughly, the mixture was allowed to stand, and was examined in 3 hours. If no coagulum could be detected the tubes were re-examined the following morning. Some strains produced solid clots, but in others the clot appeared in the form of a gelatinous globule which could be seen more easily by tilting the tube to a horizontal position. Occasionally the clot appeared as an opaque disc which could be detected on careful inspection from underneath the tube. Any degree of clotting at either time was considered positive.

Crystal violet agar. A loopful of the culture from solid medium was streaked for 1 to 2 cm. on the surface of a plate of Bacto crystal-violet agar (Chapman and Berens 1935, and Chapman 1936) and incubated 36 to 48 hours. Orange or deep violet growths were considered positive. In some instances, a strain that produced an orange growth on isolation gave a violet color when retested a few days later. Sometimes there was a fringe of violct around the orange center. Non-pathogenic strains produced white or pale violet growths. In some instances, the white growth was surrounded by a violet fringe, or was mottled with violet. The color of growths was not characteristic after prolonged incubation or storage.

Brom-thymol-blue agar. A loopful of the culture was streaked across the surface of a plate of alkaline brom-thymol-blue lactose agar (Chapman *et al.*, 1937a) and incubated for 36 to 48 hours. Pathogenic staphylococci grew luxuriantly, while most non-pathogenic staphylococci failed to grow. If the culture was seeded too heavily, non-pathogenic strains grew slightly. The color of the colonies did not seem to be significant.

Fermentation of mannitol. The culture was streaked across the surface of a plate of Bacto phenol-red mannitol agar and incubated overnight. Growths surrounded by yellow zones were considered mannitol-positive.

Fermentation of lactose. The culture was streaked across the surface of a plate of Bacto phenol-red lactose agar and incubated overnight. Growths surrounded by yellow zones were considered lactose-positive.

TABLE 1

Tests of staphylococci received from Dr. John E. Blair

STRAIN NUMBER	BLAIR			MAJOR DISSOCIANT							MINOR DISSOCIANT								SOURCE	DATE ISOLATED
	P	H	C	P	H	C	V	B	M	L	P	H	C	V	B	M	L	Per cent		
Osteomyelitis																				
191	+	+	+	+	+	+	+	+	+	+	+	0	±	+	+	+	+	5	Osteomyelitis	Jan.-Oct. 1932
201	+	+	+	+	+	+	+	+	+	+*									Osteomyelitis	Jan.-June 1935
262	+	+	+	+	⊕	+	+	+	+	+									Osteomyelitis	Jan.-June 1935
270	+	+	+	+	+	+	+	+	+	+									Osteomyelitis	July-Dec. 1935
271	+	+	+	+	+	+	+	+	+	+	+	⊕	+	+	+	+	+		Osteomyelitis	July-Dec. 1935
274	+	+	+	+	+	+	+	+	+	+									Osteomyelitis	July-Dec. 1935
283	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	10	Osteomyelitis	July-Dec. 1935
285	+	+	+	+	⊕	+	+	+	+	+	+	0	+	+	+	+	+	20	Osteomyelitis	July-Dec. 1935
290	+	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+	+	10	Osteomytlitis	July-Dec. 1935
302	+	+	+	+	+	+	+	+	+	+*									Osteomyelitis	July-Dec. 1935
328	+	+	+	+	+	+	+	+	+	+	+	⊕	+	+	+	+	+		Osteomyelitis	July-Dec. 1935
370	+	+	+	+	+	+	+	+	+	+	0	+	0	+	+	+	+	20	Osteomyelitis	July-Dec. 1936
386	+	+	+	+	⊕	+	+	+	+	+									Osteomyelitis	July-Dec. 1936
403	+	+	+	+	+	+	+	+	+	+	±	0	+	+	+	+	+	10	Osteomyelitis	Jan.-Feb. 1937
404	+	+	+	+	+	+	+	+	+	+									Osteomyelitis	Jan.-Feb. 1937
321	+	+	+	0	⊕	+	+	+	+	+	±	⊕	+	+	+	+	0	5	Osteomyelitis	July-Dec. 1935
268	+	+	+	0	+	+	+	+	+	+	0	0	0	+	+	+	+	5	Osteomyelitis	July-Dec. 1935
301	+	+	+	0	0	+	+	+	+	+	0	+	+	+	+	+	+	10	Osteomyelitis	July-Dec. 1935
											0	0	+	+	+	+	+	10		
231	+	+	+	+	0	+	+	+	+	+									Osteomyelitis	Jan.-June 1935
321	+	+	+	+	0	+	+	+	+	+									Osteomyelitis	July-Dec. 1935
255	+	+	+	0	0	+	+	+	+	0	0	0	0	+	+	+	0	20	Osteomyelitis	Jan.-June 1935
162	+	0	+	0	0	+	+	+	+	+									Osteomyelitis	Jan.-Oct. 1932
300	+	0	+	0	0	+	+	+	+	+*									Osteomyelitis	July-Dec. 1935
198	+	+	+	0	0	0	+	+	+	+									Osteomyelitis	Jan.-June 1935
143	+	0	+	+	0	+	+	+	+	+									Osteomyelitis	April 1931
181	+	0	+	+	0	+	+	+	+	+									Osteomyelitis	Jan.-Oct. 1932
142	+	0	+	+	0	+	+	+	0	+									Osteomyelitis	April 1931
209	+	0	+	+	0	+	+	+	+	+									Osteomyelitis	July-Dec. 1935

308	+	+	+	+	⊕	+	+	+	+	+	±	+	+	+	+	+	+	50	Abscess	July–Dec. 1935
200	+	+	+	+	+	+	+	+	+	+*	+	0	+	+	+	+	+	10	Abscess	Jan.–June 1935
201	+	+	+	+	⊕	+	+	+	0	+*									Abscess	Jan.–June 1935
203	+	+	+	+	⊕	+	+	+	+	+*									Abscess	Jan.–June 1935
279	+	+	+	+	+	+	+	+	+	+									Abscess	July–Dec. 1935
337	+	+	+	+	⊕	+	+	+	+	+*									Carbuncle	Jan.–June 1936
352	+	+	+	+	⊕	+	+	+	+	+									Furuncle	Jan.–June 1936
353	+	+	+	+	⊕	+	+	+	+	+*									Carbuncle	Jan.–June 1936
361	+	+	+	+	+	+	+	+	+	+	0	⊕	0	+	+	+	+	20	Pustular acne	Jan.–June 1936
369	+	+	+	+	+	+	+	+	+	+	+	⊕	+	+	+	+	+	10	Carbuncle	Jan.–June 1936
382	+	+	+	+	+	+	+	+	+	+	+	⊕	+	+	+	+	+	10	Carbuncle	July–Dec. 1936
319	+	+	+	+	0	+	0	+	0	+*									Cellulitis	July–Dec. 1935
390	+	+	+	+	⊕	+	+	+	+	+									Cellulitis	July–Dec. 1935
331	+	+	+	+	⊕	+	+	±	+	+*									Septicemia	July–Dec. 1935
351	+	+	+	0	+	+	+	+	+	+	0	0	+	+	+	+	+	10	Septicemia	Jan.–June 1936
402	+	+	+	+	+	+	+	+	+	+									Septicemia	Jan.–Feb. 1937
364	+	+	+	0	⊕	+	+	+	+	+	0	0	+	+	+	+	+	20	Septicemia	Jan.–June 1936
265	+	+	+	+	+	+	+	+	+	+	0	0	0	0	+	+	+	50	Septicemia	Jan.–June 1936
290	+	+	+	+	⊕	+	+	+	+	+*									Septicemia	July–Dec. 1935
297	+	0	+	+	0	+	+	+	+	+*									Septicemia	July–Dec. 1935
273	+	+	+	0	0	0	+	+	+	+									Post-op. infect.	July–Dec. 1935
303	+	+	0	+	+	+	+	+	+	+	+	⊕	+	+	+	+	+	10	Operative wound	July–Dec. 1935
232	+	+	0	0	0	0	0	+	0	0									Operative wound	Jan.–June 1935
233	+	+	0	0	0	0	0	0	0	0									Operative wound	Jan.–June 1935
243	+	+	0	0	0	0	0	0	0	+									Operative wound	Jan.–June 1935
258	+	+	0	0	0	0	0	0	0	+									Operative wound	Jan.–June 1935
231	0	0	0	0	0	0	+	+	+	0									Operative wound	Jan.–June 1935
399	+	+	0	+	0	0	+	+	0	+									Skin	Jan.–Feb. 1937
295	+	+	0	±	⊕	0	0	+	0	+*									Skin	July–Dec. 1935
241	0	+	0	0	0	0	0	0	0	+									Basin	Jan.–June 1935
242	0	+	0	0	0	0	0	0	0	+									Silk suture	Jan.–June 1935

* Indicates that a duplicate series of tests gave similar results.

Order of in vitro tests in tables 1 to 6: P, pigment; H, hemolysis; C, coagulase; V, crystal-violet agar; B, brom-thymol-blue agar; M, fermentation of mannitol; L, fermentation of lactose.

⊕ Indicates a non-hemolytic colony which gave hemolytic transplants.

Tables 1 to 5 show the results of tests made by the donors of the strains, also the reactions of the strains and variants as determined by us within a few days after receiving them. Cultures isolated in our laboratories are listed in table 6. In this paper the symbols P H C V B M refer to pigment, hemolysis, coagulase, crystal-violet agar, brom-thymol-blue agar, and mannitol fermentation tests, respectively.

TABLE 2

Tests of staphylococci isolated from cows with bovine mastitis by Dr. Wayne N. Plastridge

STRAIN NUMBER	PLASTRIDGE*			MAJOR DISSOCIANT							MINOR DISSOCIANT								DATE OF ISOLATION	CHARACTERISTICS OF MILK	
	H	C	M	P	H	C	V	B	M	L	P	H	C	V	B	M	L	Per cent		Leucocytes†	Cl. per cent‡
S 347	4+	+	+	+	+	+	+	+	+	+									7/ 9/35	1,900,000	0.176
S 393	4+	+	+	+	+	+	+	+	+	+§									7/17/35	1,900,000	0.223
S 905	2+	0	+	+	+	+	+	+	+	+§									12/17/36	10,000,000	
S 899	3+	+	+	+	+	+	+	+	+	+	±	0	0	+	+	0	0	4	12/17/36	2,000,000	
S 346	2+	0	+	+	0	+	+	+	+	+									7/ 9/35	3,000,000	0.179
S 440	1+	0	+	±	0	+	0	0	+	+	±	0	0	0	0	+	+	20	7/17/35	1,500,000	0.22
S 375	0	0	+	+	+	0	+	+	+	+	+	0	0	+	+	+	+	10	7/17/35	100,000	0.150
S 333	0	0	+	0	0	0	+	+	+	+									7/ 9/35	100,000	0.095

* H, hemolysis of cow's blood agar; C, coagulation of cow's plasma; M, mannitol fermentation, determined in the fall of 1935 (except S 899 and S 905).

† A leucocyte count in excess of 1,000,000 is generally regarded as suggestive of udder inflammation.

‡ A chloride content of 0.14 per cent or less is frequently referred to in the literature as normal.

§ Duplicate tests gave similar results.

Strains S 905 and S 899 were from cows whose milk appeared abnormal and reacted positively to the brom-thymol-blue test of Plastridge. Other milk samples appeared normal and gave negative brom-thymol-blue tests.

DEGENERATION OF STRAINS

Many of the strains contained variants which lacked one or more properties possessed by other variants from the same culture. Discussion will be simplified if the variant containing the fullest complement of *in vitro* properties be considered the "major pathogenic variant." It should resemble the parent pathogenic strain more closely than other variants.

From a study of dissociants it is possible to visualize the steps by which a highly pathogenic staphylococcus degenerates until it becomes devoid of pathogenic properties. On isolation it reacts positively to tests for pigment, hemolysis, coagulase, crystal-

TABLE 3

Comparative tests of staphylococci isolated from pathologic sources by Dr. Luther Thompson

STRAIN	SOURCE	ORIGINAL REACTIONS*								PRESENT TESTS							VARIANT								DATE ISOLATED
		S	H	V	B	M	L	S	G	P	H	C	V	B	M	L	P	H	C	V	B	M	L	%	
Limmer	Blood	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+									Jan.-Mar. 1937
Krall	Urine	+	+	0	+	+	+	+	+	+	+	+	+	±	+	+									Jan.-Mar. 1937
Casey	Blood	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+									Jan.-Mar. 1937
Cameron	Urine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+									Jan.-Mar. 1937
Iowa	Food Poisoning	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+									Jan. 1936
Oliver	Ureteral stone	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	+	+	+	+	90†	Jan.-Mar. 1937
Beck	Tibia	+	0	+	+	+	+	+	+	+	0	+	+	+	+	+									Jan.-Mar. 1937
Wandel	Wound	0	0	0	+	+	+	+	+	+	0	+	+	+	+	+									Jan.-Mar. 1937
Childester	Blood									+	0	+	+	±	+	+									Jan.-Mar. 1937
Schwager	Urine	0	0	+	+	+	+	+	0	0	0	0	+	+	+	+									Jan.-Mar. 1937

* Tests reported by Thompson:

SYMBOL USED	TEST	RESULT REPORTED BY DR. THOMPSON	OUR INTERPRETATION OF HIS RESULTS
S	Size of cells	*Staphylococcus aureus*	+
		Micrococcus	0
H	Hemolysis	Hemolytic	+
		Not hemolytic	0
V	Crystal-violet agar	Pathogenic	+
		Not pathogenic	0
B	Brom-thymol-blue-agar	Pathogenic	+
		Not pathogenic	0
M	Mannitol fermentation	+	+
L	Lactose fermentation	+	+
S	Sucrose fermentation	+	+
G	Gelatin liquefaction	Liquefied	+
		Not liquefied	0

† It is possible that one of the intermediate variants was isolated and that the parent strain was similar to the variant comprising 10 per cent of the colonies in the culture.

violet agar, brom-thymol-blue agar, and fermentation of mannitol and other carbohydrates but non-hemolytic variants soon appear. Loss of hemolysis is the commonest form of degeneration. Dudgeon and Goadby (1930) considered that it was related to

TABLE 4

STRAIN	SOURCE	MAJOR DISSOCIANT							MINOR DISSOCIANT							
		No.	P	H	C	V	B	M	No.	P	H	C	V	B	M	[illegible]
Staphylococci received from Mr. George J. Kupchik																
FP10	Raw milk (isol. by Dr. C. E. Dolman)	260	+	+	+	+	+	+	235	±	0	+	0	+	+	30
137	Abscess thigh (isol. by Miss Henry)	237	±	+	+	+	+	+	259	0	0	+	+	+	+	40
N[3]	Normal nasal mucous membrane	239	+	+	+	+	+	+	256	+	0	+	+	+	+	30
PF8 (E. Al.)	Raw milk, apparently healthy cow (isol. by Dr. C. E. Dolman)	241	0	0	+	0	+	+								
16 W	Wash water used for drinking glasses (isol. by Miss Rose L. Epner)	243	0	0	0	0	0	0								
LV9	Vaginal discharge (isol. by Miss Woolf)	244	0	0	0	0	0	0	262	0	0	0	0	0	0	20
Staphylococci received from Dr. R. V. Stone																
S 66	Raw milk, "random isolation"	166	±	+	+	+	+	+								
S 15	Osteomyelitis	164	±	0	+	+	+	+								
S 65	Abscess elbow	159	0	0	0	0	0	0								
S 81	Raw milk, "random isolation"	150	+	+	+	+	+	+								
Staphylococci received from Dr. H. J. Shaughnessy																
Air-5		212	0	0	0	0	0	0								
Staphylococci received from Dr. Louis Gross																
	Carbuncle		+	+	+	+	+	+								
	Pustular acne		0	0	0	0	0	0								

TABLE 5

Results of tests of staphylococci received from Dr. G. M. Dack

STRAIN	SOURCE	P	H	C	V	B	M
Staphylococcus aureus no. 10	Cervical ulcer	+	+	+	+	+	+
Staphylococcus aureus no. 24	Osteomyelitis	+	+	+	+	+	+
Staphylococcus aureus no. 27	Osteomyelitis	0	+	+	+	+	+
Staphylococcus aureus no. 33	Osteomyelitis	+	+	0	+	0	+
Staphylococcus aureus no. 40	Osteomyelitis	+	0	+	+	+	+
Staphylococcus aureus no. 49	Osteomyelitis	+	+	+	+	+	+

loss of pigment. However, the results of the present investigation show that the two are independent and that pigment is more stable (table 7).

TABLE 6

Staphylococci isolated in Clinical Research Laboratory

SOURCE	NUMBER OF STRAINS	P	H	C
Abscesses	4	+	+	+
	3	0	+	+
	1	0	+	0
	4	0	0	0
Fistula	1	+	0	+
	1	+	0	0
	1	0	0	0
Fissure	1	+	+	+
Ulcer	2	+	+	+
	1	+	0	+
	1	0	0	0
Acne	1	0	+	+
	7	0	0	0
Ear discharge	1	0	+	+
	1	+	0	0
	4	0	0	0
Rabbit eye	5	0	0	0
Antral washing, nasal sinus, ethmoid and sphenoid	21	+	+	+
	3	+	0	+
	1	0	+	+
	9	0	0	0
Infected chalazion	1	+	+	+
Carbuncle, furuncle	17	+	+	+
	4	+	0	+
	2	+	0	0

LABORATORY NUMBER	SOURCE	P	H	C	V	B	M
5395	Carbuncle	+	+	+	+	+	+
5388	Ethmoid	+	+	+	+	+	+
5246	Antrum	+	+	+	+	+	+
5302	Septicemia	+	+	+	+	+	+
5281	Septicemia	+	+	+	+	+	+
5372	Conjunctivitis	+	+	+	+	+	+

The coagulating property is still more stable, and this caused Cruikshank (1937) to believe that it did not disappear with age.

However, we have obtained non-coagulating variants from coagulating cultures.

After the coagulating power has been lost, strains may still possess crystal-violet agar, brom-thymol-blue agar, and carbohydrate-fermenting properties. Doudoroff (1936) found that, in paired dissociants, the crystal-violet agar property was more stable than hemolysis and pigment. Dudgeon and Goadby (1930) isolated non-hemolytic variants which still retained their power to ferment carbohydrates, and Pinner and Voldrich (1932)

TABLE 7

Comparison of in vitro and in vivo tests of staphylococci

RABBIT, INTRADERMAL	NUMBER OF STRAINS	RABBIT, INTRAVENOUS		PER CENT OF STRAINS REACTING POSITIVELY TO						
		Number	Per cent died	Pigment	Hemolysis	Coagulase	Crystal-violet agar	Brom-thymol-blue agar	Mannitol fermentation	Lactose fermentation
++++	16	5/5	100	94	91	97	100	91	100	100
+++	16	6/6	100	78	56	100	94	94	100	100
++	7	2/3	67	64	36	100	57	78	100	100
Total pathogenic strains........	39	14	93	82	67	99	90	91	100	100
+	5	2/3	67	40	40	0	50	70	40	75
0	28	2/10	20	30	28	4	57	61	43	81
Total non-pathogenic strains....	33	13	38	32	30	3	56	65	42	80

obtained lactose-fermenting, mannitol-fermenting dissociants which had lost their hemolytic and coagulating properties. Crystal-violet agar, brom-thymol-blue agar, and mannitol-fermenting properties are lost at about the same rate. In Pinner and Voldrich's (1932) series, lactose- and mannitol-positive parent strains produced lactose- and mannitol-negative dissociants. Lactose-fermenting power is retained longest of all. Many of Hoffstadt and Youmans' (1934) non-virulent undissociated strains fermented lactose but not mannitol.

These different steps in the degenerative process will be more clearly understood by referring to figures 1 and 2.

CORRELATION OF IN VITRO PROPERTIES WITH SOURCE OF THE CULTURES

The controversy as to the relative merits of animal inoculation experiments as compared with data based on source of the culture is typified by the statements of Julianelle (1937) who deprecated

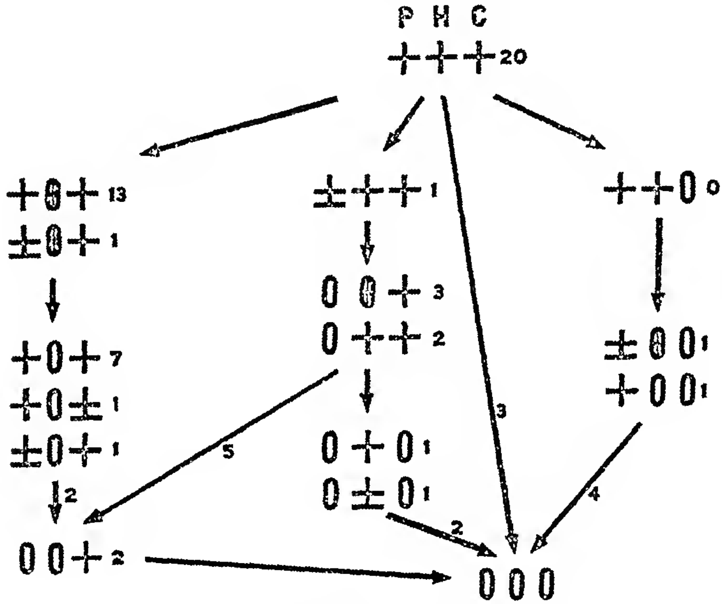

FIG. 1. DISSOCIATION OF PIGMENT, HEMOLYTIC AND COAGULATING PROPERTIES OF STAPHYLOCOCCI

Numbers after the symbols refer to the number of variants found

the former, and Thompson and Khorazo (1937) who considered the latter unreliable. We shall try to show that, when interpreted according to principles discussed in this paper, the results of *in vitro* tests are parallel with those based on the source of the cultures and on pathogenicity for healthy rabbits.

Cultures were classified as follows: (1) strains isolated from pathologic sources and not expected to be contaminated; (2) strains isolated from pathologic sources but which may have been

contaminated by staphylococci, which were non-pathogenic or only feebly pathogenic, or where there was a possibility that staphylococci may not have been the cause of the pathologic condition; and (3) cultures not expected to contain pathogenic staphylococci.

Variants reacting positively to the combination of pigment, hemolysis and coagulase (PHC) tests proposed by Chapman

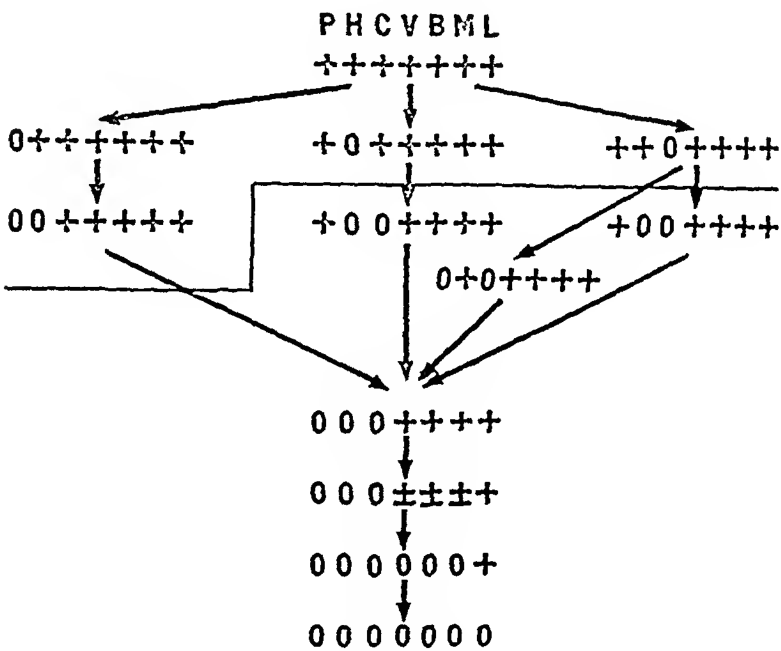

FIG. 2. DISSOCIATION OF PIGMENT, HEMOLYSIS, COAGULASE, CRYSTAL VIOLET AGAR, BROMTHYMOL BLUE AGAR, MANNITOL AND LACTOSE FERMENTING PROPERTIES OF STAPHYLOCOCCI

Variants above the line are pathogenic. Those below the line are non-pathogenic.

et al. (1934) (coagulating strains, also hemolytic aureus strains) were obtained in 97.8, 64.6 and 22.7 per cent of groups 1, 2 and 3, respectively (table 8). Cultures from raw milk should be eliminated from the third group, for reasons which will be discussed later. This would reduce the proportion of PHC positive cultures in group 3 to 5.5 per cent.

TABLE 8

Relation between in vitro tests of staphylococci and the source from which they were isolated

	IN VITRO REACTIONS*					
	+++	+0+	0++	00+	++0	All others
Group 1 (139 strains). Probably uncontaminated. Pathogenic staphylococci expected:						
Furuncle	1	4				2
Carbuncle	23					
Pustular acne	1					
Infected chalazion	1					
Septicemia	10	2				
Cellulitis	2					
Bovine mastitis (abnormal milk)	4	2				
Food poisoning	49	1	1			
Pustular acne	1					
Osteomyelitis	24	9	1		1	
Total	116 (83%)	18 (13%)	2 (1.4%)		1	2
Total PHC positive in group	136 (97.8%)					
Group 2 (82 strains). May have been contaminated, or possible error of bacteriologic diagnosis. Pathogenic staphylococci expected:						
Abscess	10		3			
Post-operative infection	1					
Wound, operative wound			1		5	1
Urine	2					1
Ureteral stone	1					
Cervical ulcer	1					
Mastitis (normal milk)					1	1
Fistula		1				2
Fissure	1					
Ulcer	2	1				1
Acne			1			8
Paranasal sinuses	23	3	1			9
Conjunctivitis	1					
Total	42 (51%)	5 (6%)	6 (7%)		6	23
Total PHC positive in group	53 (64.6%)					

* Order of tests: Pigment, hemolysis, coagulase.

TABLE 8—*Concluded*

	IN VITRO REACTIONS*					
	+ + +	+ 0 +	0 + +	0 0 +	+ + 0	All others
Group 3 (22 strains). Pathogenic staphylococci not expected:*						
Raw milk	3			1		
Wash water (drinking glasses)						1
Vaginal discharge						1
Air						1
Skin					2	
Basin						1
Silk suture						1
Ear discharge			1			5
Normal rabbits' eyes						5
Total	3		1	1	2	15
Total PHC positive in group	5(22.7)—excluding milk strains 1(5 5%)					

CORRELATION OF IN VITRO PROPERTIES WITH PATHOGENICITY FOR RABBITS

A number of factors affect the accuracy of animal inoculation experiments. For example, intravenous injections of streptococci showed marked inconsistencies when rabbits were purchased from the supply house, but consistent results were obtained with rabbits of better quality, free from snuffles, coccidiosis, etc. Pathogenic strains killed 81 per cent of supply-house rabbits, as contrasted with 27 per cent of healthy rabbits. Non-pathogenic streptococci killed 33 per cent of supply house rabbits but healthy rabbits were not affected. Similar results were obtained with staphylococci, but the series completed to date is too small for comparison and the work has been delayed by difficulty in obtaining better quality rabbits.

Selected strains were injected intradermally into healthy, 11-weeks-old, snuffle-free albino rabbits, using 0.1 cc. of an overnight brain-heart-infusion culture, and using a new rabbit for each test. The results, read at the end of 7 days, were interpreted as follows:

Elongated necrosis (at least 4.0 x 1.5 cm.) which follows the lymphatics	++++
Minimum reddening and swelling of 2.5 cm. with oval necrotic area of 1.5 x 1.0 cm.	+++
Reddening and swelling of 1.5 cm. with necrosis of 0.5 cm.	++
Reddening and swelling of 1.0 cm. with necrosis of 0.2 cm.	+
Lesser inflammatory changes	0

There was a parallel between the intradermal, intravenous (supply house rabbits), pigment and hemolysis tests (table 7). In tests of 36 cultures Cruikshank (1937) killed rabbits with 91 per cent of α lytic aureus, 83 per cent of α lytic albus variants, and 0 per cent of non-lytic albus of saprophytic origin.

The coagulase test, on the other hand, showed a sharp distinction. It was positive in groups reacting ++++, +++ and ++ but negative in those giving either + or negative intradermal tests. Although this suggests a relationship between coagulase and necrotoxin, Cruikshank (1937) concluded that they were unrelated.

CONSIDERATIONS OF THE COAGULASE TEST

(Results reported in the previous section suggest that, with few exceptions, any coagulating strain is pathogenic, while non-coagulating strains are non-pathogenic, which is in agreement with the conclusion of Hallman (1937) and Cruikshank (1937) and in harmony with the findings of previous investigators. However, no parallel has been shown between the degree of coagulating power and the degree of pathogenicity of strains.)

Errors in the coagulase test can be traced to at least three sources: the use of plasma from unsuitable animal species; the time of observing the results; and failure to recognize a minute coagulum.

The different results recorded in table 2 were traced to the use of cow's plasma in one laboratory and human plasma in the other. Further tests on 145 strains showed that the results were similar in only 78 per cent. Human plasma was positive but cow's plasma was negative in 20 per cent, while the reverse was true in 2 per cent of the cultures. Differences of a similar nature were found by Plastridge (personal communication). Walston

(1936) concluded that the power to clot plasma of different animals was characteristic for each culture.

Most investigators have used either human or rabbit plasma. Cruikshank (1937) used human plasma and maintained that rabbit plasma was next best. Fisher (1936) noted quantitative differences. We obtained qualitatively similar results in 98.7 per cent of 393 strains. There was one significant difference, however. Most strains clotted rabbit plasma firmly in less than 3 hours but many of the clots disappeared almost completely after standing overnight. Human plasma, on the other hand, was clotted more firmly after overnight incubation. A few strains did not conform to these generalities. Two per cent of the cultures gave better results with human plasma in 3 hours, while 5 per cent gave better results with rabbit plasma after standing overnight.

Although most investigators incubated the tubes at 37°C., Fisher (1936) obtained similar results at room temperature.

The coagulase test gives relatively constant results. Chapman *et al.* (1937a) showed that, when 173 strains were retested 1 month after isolation, 96.6 per cent of the reactions were unchanged. Hallman (1937) in similar experiments obtained constant results in 94.4 per cent of 233 strains.

SIGNIFICANCE OF PLATE HEMOLYSIS

Based on the finding that, in non-coagulating *Staphylococcus albus* strains, those which produced hemolysis were not more lethal for rabbits than those which did not, but that in non-coagulating *Staphylococcus aureus* strains, those which produced hemolysis killed rabbits within 2 days, Chapman *et al.* (1934) concluded that hemolysis was useful as a supplementary test of pathogenicity *only in the case of aureus strains.* Unfortunately, Thompson and Khorazo (1937) and Hallman (1937) did not classify their hemolytic reactions on the basis of pigment. It appears that Cruikshank (1937) also failed to recognize the significance of the relationship because he dismissed plate hemolysis on the ground that saprophytic strains were hemolytic.

Differentiation of hemolytic from "non-hemolytic" variants.

Hallman (1937) found that about 91 per cent of 480 strains were hemolytic on *human* blood agar plates. Thompson and Khorazo (1937) found that 96 per cent of 78 type A strains and 89 per cent of 193 strains of other types were hemolytic. On the other hand, Chapman *et al.* (1934) claimed that only 51 per cent of 690 aureus strains and 13.4 per cent of 1852 albus strains were hemolytic on rabbit-blood agar plates. In a subsequent series of 4081 strains, 67.5 per cent of aureus strains and 10.8 per cent of albus strains were hemolytic. Such serious discrepancies must be due to some vital difference in technic or in interpretation.

A possible explanation is suggested by the statement of Thompson and Khorazo (1937) that "any trace of hemolysis" was considered evidence of hemotoxin production in toxin broth. It is possible that any strain producing a trace of hemolysis on plates was also considered hemolytic. This would account for the high proportion of positive results obtained by Hallman (1937) and Thompson and Khorazo (1937). If Thompson (1932) used some intermediate criterion of hemolysis, it would account for his finding that 90 per cent of strains from pyogenic infections and 24 per cent of skin cocci were hemolytic.

Unfortunately, Chapman *et al.* (1934) did not state the degree of hemolysis which was considered positive by them, although strains giving ++ hemolysis were included in the non-hemolytic strains in their table 2, and a summary of the hemolytic properties in their table 7 carried a heading "Hemolysis (++++ and +++)." The strains were incubated overnight on rabbit blood agar and the zones of hemolysis were graded into 0, ++, +++ and ++++. Only those producing +++ and ++++ hemolysis were considered positive. If all degrees of hemolysis were considered positive by other workers, this would account for extreme differences in results.

Thompson and Khorazo (1937) explained the increased sensitivity of plate hemolysis over hemotoxin production on the basis that blood agar is a more favorable medium for the production of hemotoxin. This would make plate hemolysis tests too sensitive, and, therefore, slight degrees should be ignored.

Importance of rabbit blood in tests for plate hemolysis. Most

workers failed to state the kind of blood used. Hallman (1937) used human-blood agar but Dolman (1932) among others showed that rabbit-blood agar gave more consistent results.

Hemolysis and pathogenicity. Dolman (1932) showed that the hemolytic activity on rabbit-blood agar plates could be suppressed by antihemolytic serum. Thompson and Khorazo (1937) maintained that the hemotoxins produced by B and C strains on agar plates are identical with type A toxin. This supports the claim that plate hemolysis is related to pathogenic factors.

Plastridge *et al.* (1936) observed a relationship between plate hemolysis (cow's blood) and invasive power of mastitis staphylococci. Hemolytic cultures were associated with a high leucocyte count. (Cruikshank (1937) concluded that α lysin, coagulase and necrotoxin are present only in the pathogenic members of the species. He stated further that "coagulase and α lysin production are the two properties specifically associated with *actively* pathogenic strains" (italics ours). Dudgeon and Goadby (1930) found that the hemolytic cultures were more virulent than non-hemolytic variants obtained from them. However, non-hemolytic cultures may retain pathogenicity, as shown in Burky's (1933) experiments with strain Ha.)

The presence of both hemolytic and non-hemolytic variants in many of the cultures examined in the present series accounts for some of the errors in reports of hemolysis tests. For example, Thompson's strain Oliver was reported by him as non-hemolytic but, when the culture was plated on rabbit-blood agar, 90 per cent of the colonies were non-hemolytic. The rest were hemolytic and gave reactions typical of a major pathogenic variant, or hemolytic parent culture (see table 3).

Thus, the plate hemolysis test, as defined in this paper, is useful for determining the *degree* of pathogenicity of strains or variants.

CONSIDERATIONS OF THE CRYSTAL-VIOLET AGAR TEST

Some workers have reported difficulty in interpreting results of tests with crystal-violet agar and brom-thymol-blue agar.

Some of the color combinations are difficult to describe. The discrepancies between results reported in table 3, e.g., may be the result of such misunderstanding. The authors will be glad to furnish strains with characteristic reactions, or to examine strains from other laboratories. Personal conferences are most desirable. Only strains which produce orange colored growths, orange surrounded by deep violet, or deep violet growths are considered positive. Pale violet, or white mottled with violet are considered negative. Sometimes the white center may be overlooked on casual inspection, but it is easily differentiated from the orange center of some positive strains.

Chapman and Berens (1935) reported agreement between PHC tests and the crystal-violet agar reaction in 92.6 per cent of 594 strains. Chapman *et al.* (1937a) found agreement between PHC and crystal-violet agar tests in 86.4 per cent of 701 aureus strains and 95.7 per cent of 1012 albus strains, or 91.8 per cent of the 1713 strains. In a subsequent series, there was agreement between PHC tests and the crystal-violet agar reaction in 91.8 per cent of 281 unclassified strains. Hallman (1937) obtained a correlation between coagulase and crystal-violet agar tests in 88.03 per cent of 142 strains.

CONSIDERATIONS OF MANNITOL FERMENTATION

Innumerable reports have appeared in which the relation of mannitol fermentation to pathogenicity was discussed, but very few of them presented significant data on pathogenicity or animal inoculation tests.

The technic was not described in many of the articles and this made it difficult to appraise the reports. The incubation period varied considerably. Hallman (1937) made no mention of the medium or the indicator used but stated that many of the strains were incubated from 2 to 7 days before fermentation occurred. Thompson and Khorazo (1937) grew strains in mannitol broth for 3 days and added brom-thymol-blue indicator. Julianelle (1937) apparently used 1 week's incubation but did not state the method used. Because all necrotoxic strains (table 7) and, with few exceptions, P H C V B M positive strains (Chapman

et al., 1937c) fermented phenol-red mannitol agar overnight, it would seem that this method is satisfactory for detecting pathogenic variants.

(In the group of pathogenic staphylococci, mannitol was fermented by 88 per cent of 50 pyogenic strains (Thompson, 1932), 78 per cent of 60 orange strains (Thompson and Khorazo, 1935), 100 per cent of 40 coagulase positive cultures (Cruikshank, 1937) and 100 per cent of 39 necrotoxic strains (table 7).

In the non-pathogenic group, mannitol was also fermented by 28 per cent of skin cocci (Thompson, 1932), 18 per cent of 39 white staphylococci (Thompson and Khorazo, 1935), 55 per cent of 20 non-coagulating variants (Cruikshank, 1937), 42 per cent of 33 non-necrotoxic strains in the present series and 10.9 per cent of strains which did not coagulate plasma in another series of 375 strains.

Hallman (1937) reported 90.97 per cent correlation with the coagulase test in 487 strains. The 375 strains just mentioned gave 86.4 per cent agreement. Julianelle (1937) obtained correlation with "pathogenicity" in about 95 per cent of 102 strains.

Fluctuations in fermentative ability of dissociants (Hoffstadt and Youmans, 1934), variations in the source of the cultures, and differences in technic would explain variations in the proportion of mannitol fermenters.

The small number of pathogenic strains which do not ferment mannitol (2.7 per cent of 375 strains on the basis of coagulase, and no necrotoxic strains) shows that mannitol-negative strains are not likely to be pathogenic. However, the reverse does not hold true of mannitol fermenters.)

CONSIDERATIONS OF MASTITIS STAPHYLOCOCCI

In table 2 are listed data on staphylococci isolated from cows with mastitis. Clinical and analytical data were kindly furnished by Plastridge. Judged by the leucocyte count of the milk, strains S 375 and S 333 would not be expected to be pathogenic. The chloride content of 0.150 per cent would suggest that strain S 375 was of the borderline type. Both these suppositions were borne out by *in vitro* and *in vivo* tests, and they agreed with

clinical data. The parallel between P H C V B M properties and characteristics of the milk and cows suggests that the hypothesis regarding degeneration of staphylococci and the correlation of *in vitro* and *in vivo* properties holds as true for strains from bovine mastitis as it does for strains from other pathologic sources.

Because similar relationships were found in staphylococci isolated from suspected sources of food poisoning outbreaks (Chapman *et al.*, 1937b), it is possible that the different *in vitro* reactions are applicable to all types of staphylococci regardless of whether they possess a gastroenterotoxin, dermonecrotoxin, α or β hemotoxin, or a leucocidin.

The high incidence of pathogenic staphylococci in raw milk, mentioned in a previous section, may be explained on the basis of the small number of strains tested, the possibility that they had been selected because of suspected pathogenicity, or the possibility that they had been derived from a pathologic source, e.g., milkers or food handlers.

APPLICATION OF IN VITRO REACTIONS TO THE DIAGNOSIS OF PATHOGENICITY OF STAPHYLOCOCCI

It was shown previously that there is a tendency for strains to degenerate, and that this tendency differs qualitatively and quantitatively with different strains. Therefore, it is important to separate possible variants. Because the commonest form of dissociation is loss of hemolytic power, and because it was often accompanied by dissociation of other properties, variants with different hemolytic properties should be separated. It may not be practical to separate other types of dissociants.

A pigmented strain is probably pathogenic in proportion to the degree of pigmentation of its colonies. However, about 13 per cent of non-pigmented strains also are pathogenic.

If a pigmented strain is hemolytic, it is probably pathogenic, particularly if it coagulates plasma also. The hemolysis test has little significance in non-coagulating albus strains. A non-hemolytic strain is probably non-pathogenic unless it is a coagulating aureus strain, in which case it is probably a degenerate pathogen.

A coagulating strain is probably pathogenic and a non-coagulating strain probably is non-pathogenic.

Strains that produce orange or deep violet growths on crystal-violet agar are probably pathogenic. However, if the crystal-violet agar test is positive, but pigment, hemolysis and coagulase tests are negative, the strain probably is non-pathogenic.

A strain which grows luxuriantly on brom-thymol-blue agar probably is pathogenic, although about 6 per cent of non-pathogenic strains also grow on this medium. With few exceptions a strain that fails to grow on brom-thymol-blue agar probably is non-pathogenic.

Strains that ferment mannitol may be pathogenic but, with few exceptions, strains that do not are non-pathogenic.

Lactose fermentation is not sufficiently specific to be of diagnostic value.

Because of inherent errors in each test it is best to use a combination of tests.

CONCLUSIONS

Degeneration of staphylococci produces mixtures of dissociants having different pathogenic properties. Therefore, it is important to separate variants before applying tests for pathogenicity.

Certain details of technic must be observed in order to obtain best results. An effort has been made to analyse possible sources of error. With appropriate technic, and by interpreting results as suggested in this paper, it is possible to obtain parallel results between certain *in vitro* tests, rabbit inoculation tests, and source of the culture.

The coagulase test is the most reliable single test for the differentiation of pathogenic from non-pathogenic staphylococci.

When used in conjunction with pigment and coagulase tests, hemolysis of rabbit-blood agar permits simple estimation of the degree of pathogenicity of a strain or variant.

Tests for crystal-violet agar, brom-thymol-blue agar, and mannitol fermentation properties are useful as supplementary

tests. The two latter are more useful for isolation than for differentiation.[2]

The authors wish to thank Difco Laboratories for supplying experimental lots of brom-thymol-blue agar and for numerous helpful suggestions. They are indebted also to a large number of scientists for their advice and coöperation.

REFERENCES

BURKY, E. L. 1933 Studies on cultures and broth filtrates of staphylococci. I. Jour. Immunol., 24, 93-114.

CHAPMAN, G. H. 1936 Specificity of the dye in the crystal-violet agar reaction of staphylococci. Jour. Bact., 32, 199-205.

CHAPMAN, G. H., AND BERENS, C. 1935 Crystal violet agar as a differential medium for staphylococci. Jour. Bact., 29, 437-448.

CHAPMAN, G. H., BERENS, C., PETERS, A., AND CURCIO, L. 1934 Coagulase and hemolysin tests as measures of the pathogenicity of staphylococci. Jour. Bact., 28, 343-363.

CHAPMAN, G. H., LIEB, C. W., BERENS, C., AND CURCIO, L. 1937a The isolation of probable pathogenic staphylococci. Jour. Bact., 33, 533-543.

CHAPMAN, G. H., LIEB, C. W., AND CURCIO, L. 1937b Isolation and preliminary differentiation of food poisoning staphylococci. Food Research. 2, 349-367.

CHAPMAN, G. H., LIEB, C. W., AND CURCIO, L. 1937c The use of bromthymol blue agar and phenol red mannitol agar for the isolation of pathogenic types of staphylococci. Amer. Jour. Clin. Path. in press.

CRUIKSHANK, R. 1937 Staphylocoagulase. Jour. Path. and Bact., 45, 295-303.

DOLMAN, C. E. 1932 Pathogenic and antigenic properties of staphylococcus toxin. Canad. Public Health Jour., 23, 125-132.

DOUDOROFF, M. 1936 Association of characters among dissociates from Staphylococcus aureus. Proc. Soc. Exper. Biol. and Med., 34, 216-7.

DUDGEON, L. S., AND GOADBY, H. K. 1930 Further observations on the staphylococci, with special reference to their hemolysins and variability. Jour. Hyg., 30, 180-195.

FISHER, A. M. 1936 The plasma-coagulating properties of staphylococci. Bull. Johns Hopkins Hosp., 59, 393-414.

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[2] Since the above was written our attention was drawn to the paper by Phillips Thygeson (Bacterial factors in chronic catarrhal conjunctivitis. I. Rôle of toxin-forming staphylococci. Arch. Ophthalmology, 18: 373-387, 1937) in which similar views are expressed as to the value of pigment, coagulase, fermentation of mannitol, and "purple growth on crystal violet agar." His failure to correlate plate hemolysis tests with the production of soluble hemolysin does not conflict with the views expressed in this paper because the plate hemolysis tests were read after an excessive incubation period (48 hours).

HALLMAN, F. A. 1937 Pathogenic staphylococci in the anterior nares: Their incidence and differentiation. Proc. Soc. Exper. Biol. and Med., 36, 789-794.
HOFFSTADT, R. E., AND YOUMANS, G. P. 1934 The genetic significance of the dissociants of Staphylococcus aureus. Jour. Bact., 27, 551-560.
JULIANELLE, L. A. 1937 Determination of staphylococcal types by fermentation of mannite. Proc. Soc. Exper. Biol. and Med., 36, 117-119.
PINNER, M., AND VOLDRICH, M. 1932 Derivation of Staphylococcus albus, citreus and roseus from Staphylococcus aureus. Jour. Infect Dis., 50, 185-202.
PLASTRIDGE, W. N., WEIRETHER, F. J., AND WILLIAMS, L. F. 1936 Observations on staphylococci associated with bovine mastitis. Jour. Bact., 31, 88.
THOMPSON, L. 1932 Morphologic and cultural study of staphylococci with special reference to source. Amer. Jour. Clinical Path., 2, 125-131.
THOMPSON, R. AND KHORAZO, D. 1935 Susceptibility to lysozyme of staphylococci. Proc. Soc. Exper. Biol. and Med., 33, 299-302.
THOMPSON, R., AND KHORAZO, D. 1937 Correlated antigenic and biochemical properties of staphylococci. Jour. Bact., 34, 69-79.
WALSTON, H. D. 1936 The clotting of plasma through staphylococci and their products. Jour. Hygiene, 35, 549-558.

THE AMERICAN TYPE CULTURE COLLECTION

The removal of the McCormick Memorial Institute to the University of Chicago made it necessary to find new quarters for the American Type Culture Collection and in October it was transferred to the Medical School of Georgetown University in Washington, D. C. Ample quarters have been provided and Dr. Mario Mollari, Professor of Bacteriology in the Medical School, has been made curator. He will be assisted by Dr. Oswald Bushnell and Mr. V. Anzulovic.

Through the coöperation of the various laboratories in Washington the number of cultures available for distribution has been greatly augmented and spirochetes and protozoa have been added.

The Collection will be revised and materially increased and a new catalog issued as soon as it can be brought up to date. Apparatus for drying by the freezing method is available and as rapidly as the routine work of distributing cultures will permit those cultures for which there is little call will be preserved in this way.

The new address is: American Type Culture Collection, Georgetown University Medical School, Washington, D. C.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN NEW YORK BRANCH

Division of Laboratories and Research, State Department of Health, Albany, November 19, 1937

The Serodiagnosis of Infectious Disease. *Augustus Wadsworth*, Division of Laboratories and Research, New York State Department of Health, Albany.

Serologic tests in the study of infectious disease involve qualitative analyses and quantitative titrations. Agglutination has definite qualitative significance in typhoid fever and other infections, depending upon the titration of the activity; further differentiation with "O" and "H" antigens forecasts lines of future investigation.

Complement fixation possesses all the sensitivity of agglutination and is not so limited in the character of the antigens that may be used with it. Precipitation is the simplest procedure. The series of comparative tests here and abroad might suggest that precipitation in general is the more satisfactory in the serodiagnosis of syphilis. The difficulty in arriving at a true evaluation, however, lies in the fact that in the qualitative determination there is lacking that accurate quantitative analysis which is necessary for diagnosis.

Quantitative procedures in complement fixation which have recently been developed are therefore most timely. The data obtained with them, which will be published in detail, justify the conclusion that in the report of the 1936–1937 series of the United States Public Health Service the results with the precipitation tests, despite the fact that the first general impression may favor them, do not quite equal those obtained with complement fixation as reported by Doctor Gilbert from this laboratory, No. 27. (Jour. Amer. Med. Assoc., 1937, 109, 425.)

Basic Principles Governing the Fixation of Complement and Their Application to Practical Tests. *Frank Maltaner*, Division of Laboratories and Research, New York State Department of Health, Albany.

Studies with bacterial or protein antigens and their homologous antisera and with tissue extract antigens and syphilitic sera demonstrated constant relationships between the specific activities of serum, antigen, and complement which provide a rational basis for the performance of complement-fixation tests in general and allow an accurate titration of the immune reaction. Thus, the change in activity of complement is directly proportional, first, to the quantity of antigen present—provided a satisfactory excess of immune serum is present—and, secondly, to the amount of immune serum, provided a satisfactory excess of antigen is present. The relationship be-

tween the amounts of antigen and immune serum which cause the same change in complement activity is constant.

The point of 50-per-cent hemolysis is the most accurate for evaluating the changes which occur in complement-activity. By interpolation of values between 10- and 90-per-cent hemolysis to that of 50-per-cent, equally accurate results are obtained. Three to five tubes suffice for titrating sera of low or moderate activity whereas highly active sera require a more extensive titration. The titer of the serum may be expressed in terms of the change in complement-activity or in direct relation to antigen.

THE QUANTITATIVE DETERMINATION OF THE FIXATION OF COMPLEMENT BY IMMUNE SERUM AND ANTIGEN: FURTHER STUDIES WITH TUBERCLE ANTIGEN AND IMMUNE SERUM. *Elizabeth Maltaner*, Division of Laboratories and Research, New York State Department of Health, Albany.

Previous studies of the fixation of complement by tubercle antigen and immune serum have been extended to include observations in the region of serum, antigen, and complement excess.

In experiments covering a wide range of complement concentration there was a direct proportion between complement and immune serum when antigen was present in excess, and between antigen and complement when immune serum was present in excess. By determining the ratios of the slopes of the straight-line graphs representing these relationships, constant values for the specific reactive capacity of antigen with immune serum were obtained.

The activity of serum and antigen in relation to each other and to complement may be accurately evaluated on the basis of the constant relations which exist between them. The complement-fixation test for tuberculosis described in 1925 is being revised on the basis of these studies to provide a quantitative determination of the reaction.

QUANTITATIVE COMPLEMENT-FIXATION TESTS WITH SPECIMENS SUBMITTED BY THE UNITED STATES PUBLIC HEALTH SERVICE IN EVALUATION OF SERODIAGNOSTIC TESTS FOR SYPHILIS. *Elizabeth Maltaner*, Division of Laboratories and Research, New York State Department of Health, Albany.

Following the official examination in the series of tests sponsored by the United States Public Health Service, the specimens were studied by a quantitative complement-fixation test. The numerical value obtained with the new method is a direct index of the titer of the serum. Titers greater than 10 are not differentiated; those less than 2 are classed as negative findings. The highest titer obtained with the control sera was 1.5. Sixty-seven of 191 syphilitic sera had titers greater than 10, 31 had titers of 7 to 10, 55 of 4 to 6, 19 of 2 to 3, and 19 of less than 2; seven of the latter were greater than 1.5.

Methods of reporting reactions as ±, +, 2+, 3+, or 4+ did not provide a reliable quantitative evaluation of the results. In general, specimens which reacted with both non-cholesterolized and cholesterolized extracts had high titers in quantitative tests with cholesterolized antigen. Reports made with different methods in the Federal series varied considerably with sera of low titer; the few disagreements observed with sera of high titer were important in indicating the danger of false or misleading reports due to prozone reactions.

Report on a Preliminary Precipitation Test as an Aid in the Serodiagnosis of Syphilis. *Rachel Brown*, Division of Laboratories and Research, New York State Department of Health, Albany.

Specimens submitted by the United States Public Health Service, 1936-37, were examined by the precipitation test of Wadsworth and Brown (Jour. Immunol., 1936, 31, 155) which is used in the New York State laboratory routinely in conjunction with an oversensitive complement-fixation test to select specimens requiring further examination. Whenever sufficient material was available after the completion of other tests, the sera which gave partial or no precipitation but definite complement fixation were retested in dilution to detect prozone reactions.

With 198 sera from cases of syphilis, precipitation was marked (2+ − 4+) with 93.9 per cent and partial (± − +) with 2 per cent. No reaction of any degree occurred with 100 specimens from nonsyphilitic cases. This was a greater number of reactions than was recorded with the same sera from cases of syphilis with any of the precipitation methods used in the control laboratories in instances where no reactions were obtained with sera from nonsyphilitic cases.

The amount of antigen originally used in the test has been doubled in order to reduce the number of prozone reactions.

Quantitative Complement-fixation Tests with Bacterial Antigens: The Gonococcus. *Christine E. Rice*, Division of Laboratories and Research, New York State Department of Health, Albany.

Specimens of human sera (447) have been titrated quantitatively for complement-fixation with two gonococcus antigens: I—a filtered extract of frozen and thawed cells; II—a broth culture filtrate. Both antigens were purified and concentrated by ultrafiltration.

Titers of 198 sera from persons with past or present history of gonorrhea (four days to forty years): Over 2.0 with both antigens, 113; over 2.0 with antigen I only, 3; over 2.0 with antigen II only, 15; below 2.0 with both antigens, 67. Thirty-eight had titers of between 5.0 to 10.0, 16 had titers of over 10.0.

Titers of 249 sera from persons with no recorded history of gonorrhea: Over 2.0 with both antigens, 32; over 2.0 with antigen I only, 1; over 2.0 with antigen II only, 19; less than 2.0 with both antigens, 197. Of these 52 reacting sera, the majority of which (35) had titers of between 2.0 and 3.0 with one or both antigens, 34 were from cases of syphilis, 3 from rheumatic fever, 2 from rheumatoid arthritis, 2 from cancer, 1 from epithelioma, 1 from erysipelas, 9 from pneumonia. Sera from 41 healthy persons did not react appreciably with either antigen.

Quantitative Complement-fixation Tests with Serum and Spinal Fluids from Meningococcus Meningitis Cases, Convalescents, and Contacts. *Grace M. Sickles and Christine E. Rice*, Division of Laboratories and Research, New York State Department of Health, Albany.

Serum from patients convalescent from three to six weeks from group-I-III meningococcus meningitis was titrated by a simplified quantitative complement-fixation technic with extracts and culture filtrates of meningococci concentrated and purified by ultrafiltration. All cases had been serum treated. Of five sera from these convalescent carriers, four reacted to

titers of 3.0 to 7.4 with extracts and filtrates of group-I-III meningococci and to a lesser degree, titers 1.2 to 2.8, with group-II filtrates; the *fifth* was of low titer, 1.9, with group-I-III extracts. The reactions of sera from two convalescents who were no longer carriers and from seven contact carriers from whom group-II or atypical group-II meningococci had been isolated, were minimal.

Spinal fluids from five cases of serum-treated meningitis were titrated for the presence of meningococcus antigen. Of three specimens from which group-I-III meningococci had been cultured, two gave no fixation, one a very strong reaction, titer 18.0, with group-I-III rabbit serum and a weak reaction with group-II rabbit serum. This patient subsequently died. The fourth specimen from which group-II meningococci had been isolated fixed complement with group-II serum, titer 7.5, but not appreciably with group-I-III serum. From a fifth specimen without significant reactivity with either group-I-III or -II sera, an atypical group-II meningococcus was obtained on culture.

A Note on the Biological Properties of Strains Isolated from Cases and Carriers in an Outbreak of Meningococcus Meningitis. *Sophia M. Cohen*, Division of Laboratories and Research, New York State Department of Health, Albany.

A comparative study of strains from patients, convalescents, and contact carriers in an outbreak of meningococcus meningitis in New York State revealed marked differences which, in this limited series, appeared to be related to the serological group and to the source of the strains.

The cultures from the eight patients were classified as group I-III by agglutination and precipitation (immune-serum-agar plate) tests. Strains from two of three convalescent carriers corresponded to those isolated during the acute stage of the disease; that from the third was related to group I-III according to the agglutination reactions but lacked precipitative activity with group I-III sera. Five of six contact carrier strains, isolated late in the outbreak, fell into group II or "X" related to II; the sixth was intermediate and showed some relationship to group I-III in agglutinative activity and to group II in precipitation reactions.

In general, among the limited number of strains tested, those from patients and convalescents were of higher virulence for mice and remained viable longer in sodium-chloride solutions than the contact carrier strains. Marked differences in toxigenic activity were not demonstrated. The atypical intermediate strains from two of the carriers, one a convalescent and one a contact, possibly represented modified group I-III strains.

Preliminary Report on the Action of Immune Sera in Conjunction with Prontosil and Sulfanilamide in Streptococcus Infection in Mice. *Augustus Wadsworth and Grace M. Sickles*, Division of Laboratories and Research, New York State Department of Health, Albany.

The Action of Bacterial Toxins on the Tissues of Cold-blooded Animals. *Myrtle Shaw*, Division of Laboratories and Research, New York State Department of Health, Albany.

Further studies of the effect of bacterial toxins on cold-blooded animals are reported. To determine the effect

of toxins on regeneration of planaria, bacteria-free worms were necessary. This was accomplished by repeated washings in sterile water, continued until sterility tests showed no bacterial contamination. These planaria were cut transversely, both halves placed either in a hanging drop, or in tubes of suitable medium containing diluted toxin. Purified diphtheria toxins did not affect the regeneration of anterior and posterior regions but crude toxins were injurious.

Tadpole heart and liver tissues were cultivated *in vitro* in frog plasma and Tyrode's solution. A like medium containing crude diphtheria toxin diluted 1:10 (50 M.L.D.) or similarly diluted crude botulinus toxin type B had no inhibitory effect on the growth.

Similar studies were made on *Fundulus* embryos, removed aseptically from the egg, at the hatching stage. Fragments of tissue were explanted in drops of medium with diluted sea water as a base. Considerable migration of cells occurred within twenty-four to forty-eight hours. Crude diphtheria or botulinus toxins did not inhibit migration if used in dilutions higher than that in which broth itself inhibited. Botulinus toxin purified and concentrated by ultrafiltration did not inhibit migration in a 1:10 dilution.

OXIDATION-REDUCTION POTENTIALS AND A METHOD OF DETERMINING THEM IN SKIN. *Calvin C. Torrance*, Division of Laboratories and Research, New York State Department of Health, Albany.

In order to determine the O/R potentials of skin, it is necessary to inject both oxidized and reduced solutions of sterile dyes. The dyes must be dissolved and sterilized by boiling immediately before use. In the method developed here, they are reduced in tubes designed to maintain anaerobic conditions and protected from light. A rubber stopper carries a glass filter which extends below the surface of the liquid and an outlet tube with cotton filter. Before sterilization, a pinch of platinized asbestos is placed in the tube and sufficient distilled water is added to raise a petrolatum seal above the filter. The sterile dye solution is added with a syringe through rubber tubing attached to the filter tube. In preparing the solution of the dye, allowance must be made for the water in the tube. Hydrogen gas is passed down the filter stem until the color of the dye is bleached. The reduced dye can then be aspirated directly through the filter into the syringe used for injection without contamination or reoxidation. Thionin, cresyl blue, gallocyanine, methylene blue, and indigo-tetrasulfonate, -trisulfonate, and -disulfonate were found useful in studies of skin.

CONNECTICUT VALLEY BRANCH

YALE MEDICAL SCHOOL, BRADY AUDITORIUM,
310 CEDAR STREET, NEW HAVEN, CONNECTICUT,
DECEMBER 4, 1937

THE FIBRINOLYTIC TEST IN CLINICAL USE. *Paul L. Boisvert*, Department of Pediatrics, School of Medicine, Yale University, New Haven, Connecticut.

The streptococcal fibrinolytic test of Tillet and Garner had promise of being a practical serological test for recent hemolytic streptococcal infection. Recently, however, Waaler has re-

ported that the test is also positive (i.e. the plasma clot is resistant) in pneumonia caused by organisms other than the hemolytic streptococcus.

The fibrinolytic test has been used clinically in our pediatric laboratory for over a year. Although in young infants variable results have been obtained, the test is generally positive in children recovering from a hemolytic streptococcal infection. We have also observed positive tests in the majority of patients in the pediatric age group with pneumococcal pneumonia. However, repeated tests on these patients have revealed that the test is positive only during the acute febrile stage of the disease, and rapidly becomes negative. This reaction is quite different from that seen in children with hemolytic streptococcal infections. In the latter the test is negative during the active infection and generally becomes positive at the time of the patient's recovery.

The results indicate that a series of tests may be of clinical diagnostic value. The simplicity and inexpensiveness of the test add to its practical value.

THE ACTION OF INTESTINAL BACTERIA ON ASCORBIC ACID (VITAMIN C). *William B. Esselen Jr.*, Department of Bacteriology and Physiology, Massachusetts State College, Amherst, Massachusetts.

The purpose of this work was to determine whether certain intestinal bacteria destroy vitamin C, and if they do, what explanation may be offered for this action. The results obtained are in contradiction to those previously reported by other workers. Intestinal bacteria (Four strains of *Escherichia coli-communis*, seven of *Escherichia coli-communior*, and one strain each of *Aerobacter aerogenes*, *Salmonella pullorum*, *Salmonella aertrycke*, *Salmonella enteritidis*, *Eberthella typhi*, *Bacillus subtilis*, and *Proteus vulgaris*) exert a protective action on the readily oxidized ascorbic acid. This protective action appears to vary directly with the suitability of the medium for bacterial growth and the numbers of organisms present. The effect may be due to: (1) increased acidity produced by the organisms, (2) their reducing power, (3) the removal of oxygen from the media by them, or (4) metabolic products produced. It was shown that the lowering of pH by the bacteria is not a factor in preventing the destruction of ascorbic acid. Furthermore, no correlation was established between the ability of the organisms to reduce methylene blue and the stability of ascorbic acid in their presence. Carbon dioxide, but not hydrogen, exerts a protective action on ascorbic acid, similar to that produced by bacteria growing in media containing readily fermentable carbohydrates. It is suggested that the ability of intestinal bacteria to retard the destructive oxidation of ascorbic acid may be due to their ability to produce carbon dioxide.

THE EFFECT OF VACCINIA IMMUNE SERUM IN REDUCING THE NUMBER OF COUNTABLE LESIONS ON THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING CHICK EMBRYO. *Elizabeth Osterman and Rachel E. Hoffstadt*, Department of Bacteriology, University of Washington, Seattle, Washington. (Miss Osterman now a graduate student in the Department of Bacteriology, Yale University, New Haven, Connecticut.)

There is little direct evidence supporting the belief that hyperimmunization of animals with vaccinia increases

the virus "neutralizing" property over that present in the serum taken after simple vaccination. An attempt was made to obtain more definite information by utilizing the method of Burnet (1936) and Koegh (1936), titrating the serum-virus mixtures on the chorio-allantoic membrane of the developing chick embryo.

Serum was collected from two rabbits fourteen days after dermal vaccination. They were then hyperimmunized by intravenous inoculation of saline extracts of dried vaccinial membranes, serum being taken after a total of 50 mg. and 150 mg. had been injected. Two control rabbits received normal membrane extracts in the same amounts. The serum-virus mixtures were inoculated into each of ten eggs, and the lesions appearing on the countable membranes averaged. The percentage reduction in the number of lesions that appeared in the presence of serum taken after vaccination and after hyperimmunization is shown in Table I.

TABLE I

Percentage reduction in number of lesions

SERUM	RABBITS			
	I	II	III	IV
Normal	0	0	—	—
Normal memb. 150 mg.	—	—	0	0
Dermal vaccination	73	57	—	—
Hyperimm. 50 mg.	88	82	—	—
Hyperimm. 150 mg.	92	83	—	—

Parallel titrations using both saline and distilled water as diluents, and observations of the mixtures both macroscopically and microscopically by dark field illumination, gave no evidence that flocculation had occurred under the conditions of the test. Therefore, flocculation could not have played a rôle in reducing the count.

The results support the belief that the "neutralizing" properties of vaccinial antiserum are increased by hyperimmunization following vaccination.

Field Studies on the Recovery of Poliomyelitis Virus. *J. R. Paul, J. D. Trask, and A. J. Vignec,* Department of Pediatrics, School of Medicine, Yale University, New Haven, Connecticut.

Recovery of poliomyelitis virus from contaminated material has always been difficult. Whether or not this depends on inadequate methods is uncertain. Kramer has described the use of ether as a satisfactory bactericidal agent. Accordingly, nasal washings and stool emulsions were treated with ether and in most instances frozen and concentrated in a Florsdorf-Mudd apparatus. Intracerebral and intraperitoneal inoculations were made into monkeys.

Three criteria were adopted as evidence of positive results: (1) Production of the experimental disease, (2) Histopathology, (3) Passage into a second animal.

Success was attained in three out of four children studied in the first day of the disease, twice from nasal washings and once from the stools. Ten attempts to recover the virus were made late in the disease with one successful result.

The high percentage of positive findings reported here would seem to indicate that the method employed has considerable promise.

The Influence of Estrogenic Hormone on the H-Ion Concentration and Bacterial Flora of the Human Vagina, with Special Reference to Döderlein's Bacillus. *Louis Weinstein and Joseph H.*

Howard, Department of Bacteriology, Yale University, New Haven, Connecticut, and The Gynecology Service, City Dispensary, Bridgeport, Connecticut.

The injection of estrogenic hormone into women in the post-climacterium led to the development of a high degree of H-ion concentration in the vaginal secretions in eight out of nine cases. The only individual who did not respond was one who had undergone ovariectomy several years previous to the time of this experiment. Studies of the histological structure of the vaginal mucosa, before and after treatment with the hormone, revealed that a low pH could be correlated, in every instance, with a growth of the mucosal tissue.

Attempts to establish a correlation between the acidity of the vaginal secretions and the presence or absence of the Döderlein bacillus failed; in seven of the nine individuals examined no organism of this type was demonstrable culturally in a pH range of 3.8 to 7.0. In one instance large numbers of the Döderlein organism were present at a pH varying from 6.6 to 7.2. This was in the ovariectomized individual who did not react to hormone treatment. In the other case the aciduric vaginal bacillus was recovered, at times in fairly large amounts, at pH 6.0 to 6.8, but it was not demonstrable at other times at a pH level which was the same or considerably lower.

It is concluded that there is very little, if any, correlation between large amounts of acid in the vaginal secretions and the presence of Döderlein's bacillus. On the basis of this fact, it is felt that this organism has little value as an indicator of vaginal health or as a therapeutic agent.

David H. Bergey.

David Hendricks Bergey[1]

1860 -- 1937

David Hendricks Bergey was born on the Mennonite meeting-house farm in Shippack township, Montgomery County, Pennsylvania, December 27, 1860. As was the custom in the rural sections of Pennsylvania in those days, he worked as a boy on the home farm during the summer time and attended school in the winter time. After he was 18, he attended private and normal schools and finally taught for two winters in rural schools before he decided to study medicine. It is interesting to note that he started his medical training in the good old fashioned way in the office of the local physician, Dr. Samuel Wolfe of Shippack.

After this, he entered the University of Pennsylvania for further scientific and medical training and secured his B.S. and M.D. degrees simultaneously in 1884 at a time when knowledge of Pasteur and Koch's recent discoveries was causing eager discussion in American medical circles. Dr. Henry Formad of the Medical School staff had made two visits to Dr. Koch's laboratory during this period, and Dr. Bergey received his first introduction to bacteria in Dr. Formad's laboratory and from his lectures on the germ theory of disease. In June, 1884, he married Anne S. Hallman of Shippack, a sister of one of his former pupils.

For nearly ten years, Dr. Bergey practiced medicine at North Wales, Pennsylvania, before he returned to the University in 1893 as a student, and later as the Scott Fellow in Hygiene in the newly built laboratory of Hygiene. In 1895 he was made Assistant in Chemistry and when Dr. Abbott was appointed Director of the Laboratory in 1896 he became First Assistant. Later (1903) he was made Assistant Professor and in 1926 Professor of Hygiene and Bacteriology. In 1903, he took charge of the teaching of bacteriology to the medical students at the Uni-

[1] President, Society of American Bacteriologists, 1915.

versity, and continued this work until he retired in 1932. During the World War Dr. Bergey served as a Major in the Sanitary Corps at Fort Oglethorpe, Georgia.

Not content to retire even at 72, Dr. Bergey became interested in the development of tetanus toxoid and other biologic products for his friend Dr. H. K. Mulford of the National Drug Company and continued at this work until his death on September 5, 1937 at the age of 76.

Dr. Bergey was a tireless investigator as well as a teacher and his studies led to the publication of a long list of scientific papers beginning with his report as a Fellow on the Nature of the Air Expired from the Lungs. His *Principles of Hygiene* which appeared first in 1901, ran through a series of seven editions, and was widely used. Bergey's *Manual of Determinative Bacteriology* has been preëminent in its field ever since it was first published in 1923. The manuscript for this book was started soon after he served as President of the Society of American Bacteriologists in 1915, at a time when the Society was active in revising the older classification outlines to fit the newer knowledge. Feeling that the work would be more satisfactorily accomplished if carried out by a group of individuals, Dr. Bergey requested the Society to appoint a committee to work with him in developing the Manual. This was done and through the publication of four successive editions under the auspices of the Society, the Manual came to have a more and more widely recognized field and authoritative standing.

It was Dr. Bergey's wish from the beginning that all profits from this book be used to develop research in the field of systematic bacteriology. As it proved difficult to carry out his plan under the auspices of the Society, the accrued royalty funds were returned to him in 1935, and he placed them in the hands of a self perpetuating Board of Trustees who have accepted the responsibility for preparing the fifth and later editions of the Manual. Thus, he has established for himself a memorial that will remain as long as competent workers can be found to carry out the trust.

The JOURNAL OF BACTERIOLOGY was established by the Society

of American Bacteriologists during the time that Dr. Bergey was President of the Society. The enterprise commanded his interest from its start and his presidential address on "The Pedagogics of Bacteriology" is the first article in the first volume of the Journal. The interest aroused by this address led him to prepare a further report on "Early Instructors in Bacteriology in the United States" which appeared in the next volume of the Journal.

It will interest his scientific colleagues to know that his hobby was genealogy. He served as Recording Secretary and Historian for the Bergey Family Association for many years and in 1925 published a 1150 page book under the title *The Bergey Family in America* giving the lineage of over five thousand descendants of John Ulrich Berge who came to America probably from Switzerland in 1717, finally settling in southeastern Pennsylvania in 1726.

As a teacher, he insisted on high standards of work and thereby won the lasting friendship and regard of a host of younger workers. By his modesty, unassuming attitude and unwillingness to enter into controversies, he won many warm friends. It is a tribute to his sterling worth to report that those that knew him best respected and admired him most. A friend has left us, his work well done; and his spirit lives on in the lives of those with whom he came in contact.

ROBERT S. BREED

MITOSIS-LIKE ACTIVITY IN BACILLUS SP.

A Preliminary Report[1]

H. L. CHANCE

Department of Botany, The University of Oklahoma, Norman, Oklahoma

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The organism used was isolated from the soil and grown on glucose agar. In size and fermentation it corresponds in a measure with *Bacillus mesentericus*. Only the R type was used since the staining reaction of the S type was not satisfactory.

A twelve- to thirteen-hour culture was used as the parent culture. Since stains of the growing culture were to be made at different time intervals, as many transfers were made as time intervals, thus giving a separate culture for each staining period.

After the transfers were made cells were removed from the parent culture and stained (figs. 1 and 2). Some of these cells stained throughout. Others had more heavily stained structures within the cytoplasmic region. The arrangement of these structures, within limits, constituted a type of configuration.

The cells from the two-hour culture (fig. 3) may or may not stain. Some of the figures resemble in a measure those characteristic of the parent cells while others may contain one or even two spherical bodies. Some of these cells stain rather uniformly throughout, while others give a motley effect. A more deeply stained spherical body may appear in cells otherwise uniformly stained. Where two spherical bodies appear they may be equal or unequal in size and occur separately or partially united. The wall region of some cells remains unstained. The staining pic-

[1] Contribution from the Department of Botany, University of Oklahoma, No. 48.

The writer is indebted to School of Pharmacy, University of Oklahoma for many chemicals; to Dr. Lloyd E. Harris and Dr. Bruce Houston for advice on chemical problems and to Dr. H. M. Hefley for photographic work.

ture at this age suggests a redistribution and reorganization of material within the cell.

One of the most striking features of the cells of the three-hour cultures (fig. 4) is the general disappearance of the type of figure characteristic of the parent cells. In comparison with the cells from the two-hour cultures there is a relative increase in the number of cells staining throughout as well as of spherical structures. Two spherical bodies appear in some cells. This condition seemingly is more pronounced where there is a tendency to chain formation. Figure 4 shows a preponderance of single spherical bodies. The central portion of some does not stain, thus giving a ring-like effect. Others show one or more small projections. The appearance of these projections and of the unstained central portion seem to occur just prior to the initiation of a general breakup of the spherical body. The time lapse from inoculation to the initiation of the breakup of the spherical body corresponds well with the "lag phase" of growth as judged by the appearance of the slopes. Little growth is visible up to this time but the close of the period ushers in a period of very rapid growth.

The breakup of the spherical bodies (fig. 5) coincides with the initiation of pronounced changes in the internal configuration of the cells. This usually occurs around the fifth hour though figure 5 shows a six-and-one-half-hour culture. As the appearance of the projections seems to forerun the breakup of the spherical bodies, an asterisk-like figure appearing in some cells may be the beginning of this process. In many cases the asterisk-like figure is made up of eight separate units.

With development these separate units probably merge into the two parallel four-beaded bars seen in some cells. A single bar having four larger bead-like structures appears in other cells. Cells without evidence of a division wall show figures made up of four units in each end. A similar configuration, but showing evidence of a division, may also be present. Again, single cells may show only the four units. This regularity does not hold for all cells as many apparently possess an irregular number. While there is a diversity of figures there is also a uniformity. The four-unit structure occurs rather frequently suggesting a definite

sequential series. Whatever the significance of the change in the internal configuration, the appearance of the period of high activity begins, parallels and declines with the "grand period" of growth.

The condition during the period of rapid growth is shown in figures 6 and 7. Though given as eight-hour cultures the condition in figure 7 is more advanced in development. An interesting feature in figure 6 is the appearance of paired dumb-bell-shaped structures in some of the cells. The four unit configuration previously mentioned is shown in figure 7.

Little attention has been given to the work after sporulation starts. However there are fewer figures and these are much heavier in outline. Shortly after sporulation starts some cells stain throughout and some of these contain more heavily stained spherical bodies. The latter structures have not been followed to observe their ultimate fate. Observation of older cultures shows that some cells do not readily sporulate, or at least that the cells retain the vegetative form. All spores do not readily take the stain as shown in figure 8.

The method of staining, though in progress over two years and repeatable under proper conditions, is not given in detail since the preparation of the mordant needs more research before the procedure is standardized. However, the theory underlying the work is given here. First, use living cells. Second, reduce the cells. Third, fix the cells in a reduced condition. Fourth, stain with an oxidizing stain. The reducing agent used was nigrosin and the stain was rosaniline hydrochloride. Both must undergo treatment before use. Other reducing agents and stains will work, as, for the latter, methylene blue, malachite green, eosin, Bismark brown, etc., but, so far, not as satisfactorily as rosaniline hydrochloride.

Theoretically, the principle of staining as outlined should be applicable to any respiratory mechanism, reducible protein or protein complex. This does not necessarily mean that one reducing agent and stain preparation can be used for all species. There is some indication of a degree of specificity as applied to different organisms.

PLATE 1

All figures magnified about 2000 times

Fig. 1. Cells from 13-hour culture. Appear physiologically older than age indicates.

Fig. 2. Cells from 13-hour culture. More nearly normal picture for age. Some cells not staining, some showing changes still going on in internal structures, others showing drift toward sporulation, particularly the four or five cells at lower right.

Fig. 3. Cells from 2-hour culture. Note resemblance of some figures to those in figures 1 and 2. Also the appearance of solid spherical bodies; stained and unstained cells which may indicate a redistribution of material.

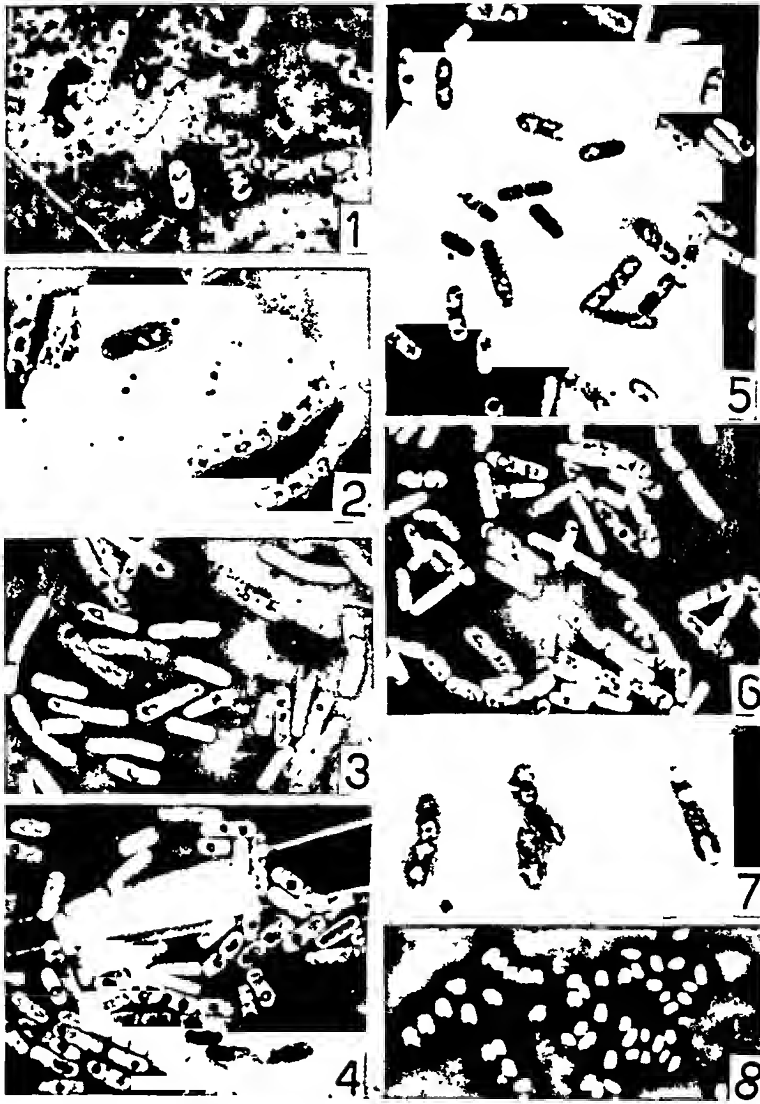
Fig. 4. Cells from 3-hour culture. The older types of figures have now disappeared with the appearance of a greater number of spherical bodies along with stained and unstained cells. Wall region unstained in some cells. Figures 3 and 4 show "lag phase" condition.

Fig. 5. Cells from 6½-hour culture. An intermediate stage between the "lag phase" and the grand period of growth. The critical stage showing stained and unstained cells, spherical bodies, and the breakup of spherical bodies. The asterisk-like figures are visible in some cells while others show a more advanced condition.

Fig. 6. Cells from 8-hour culture. Grand period of growth condition. Note at least three cells showing paired dumbbell-shaped structures.

Fig. 7. Cells from 8-hour culture showing four unit type of figure.

Fig. 8. Spores—not staining.



(H. L Chance Mitosis-Like Activity in Bacillus sp)

THE USE OF THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING CHICK EMBRYO AS A MEDIUM IN THE STUDY OF VIRUS MYXOMATOSUM[1]

RACHEL E. HOFFSTADT AND K. STEPHEN PILCHER

Department of Bacteriology, University of Washington, Seattle

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In a study of the nature of infectious myxomatosis of rabbits various workers have attempted to cultivate the causative agent. Hyde and Gardner (1933) employing sterile heparinized rabbit blood found it no longer infective after the virus has been sufficiently diluted to give negative results. Hobbs (1928) using a medium, the composition of which approximated closely that of rabbit tissue, was only able to maintain the infectivity of the virus for 8 days outside the body of the animal. On the third subculture it was no longer infective after 96 hours. The only apparent successful attempt at the cultivation of the virus was that reported by Benjamin and Rivers (1931). In a medium, the essential constituents of which were the monocytes and other cellular elements in the pleural exudate of rabbits, obtained by irritation of the pleural cavity by injections of beef broth and gum acacia, they carried the virus through 20 subcultures with an increase of original titer of 1:100 to 1:10,000 in some cases and in others 1:100,000.

Since Woodruff and Goodpasture (1931) first introduced the technic of cultivating the virus of fowlpox upon the chorio-allantoic membrane of the chick embryo, this method has been widely used in the studies of viruses. In view of the remarkable species-specificity exhibited by virus myxomatosum, it was deemed of interest to determine whether or not this virus would remain viable upon an embryonic membrane of such a distinctively alien species as that of the chicken.

[1] The study was aided by a grant from the Biological Committee of the American Academy of Arts and Sciences.

METHODS

The virus. The virus used was obtained from Dr. Roscoe Hyde of the Johns Hopkins School of Hygiene and Public Health and was of the South American strain. After two serial passages in animals, it was stored in 50 per cent glycerol at 5°C. for four months before it was used in this study. Since the virus is not easily filtrable, the following method was used in order that a bacteria-free tissue could be obtained. A number of intradermal inoculations of 0.2 cc. of a 1:20 dilution of myxomatous tissue in sterile physiological salt solution were made on the flanks of aseptically prepared rabbits. After the tumorous areas had reached 1 to 2 cm. in size and before the centers had become hemorrhagic, the animal was sacrificed and the tissue harvested with utmost care. It was then stored in 50 per cent glycerol and later tested for bacterial contaminants. About 75 per cent of the tissue salvaged was found entirely free of bacteria and the remaining 25 per cent, which was contaminated, was discarded.

The technic for inoculating membranes. The method used for inoculating the chorio-allantoic membranes with the virus suspension was essentially that employed by Woodruff and Goodpasture (1931) with slight modifications. The work was carried out in a hood free from air currents and at a constant temperature of 22°C. and hence it was not found necessary to maintain the temperature of the embryo by partial immersion in a warm water bath while operating.

The fertile eggs used throughout the experiment were from the same flock of chickens, which tested free of *Salmonella pullorum*. A dozen eggs yielded from six to ten living embryos. On the exposed chorio-allantoic membrane of four to six 10-day chick embryos, at each inoculation, 0.1 cc. of a 1:20 dilution of bacteria-free tissue extract containing myxomatosis virus was dropped. At least two embryos were used as controls. In the first experiments these were inoculated with sterile saline of the same volume as above and in subsequent studies with extracts of previous control membranes.

Staining methods. Membranes to be sectioned were fixed in bichloride formol solution and sections were stained with both

Mallory's phloxine methylene azure and Giemsa stain (Wolbach modification). Better differentiation was obtained with the Giemsa stain when 96 per cent alcohol, to which had been added 5 to 10 per cent of a saturated alcoholic solution of colophonium, was used as a differentiating agent. The same methods, as well as the modification of Wright's stain as applied by Lewis and Gardner (1932), were used in tissue smears and spreads, and that of Giemsa again was found most satisfactory.

FACTORS INFLUENCING THE VIABILITY OF THE VIRUS IN THE CHORIO-ALLANTOIC MEMBRANE OF CHICK EMBRYO

Temperature. In early experiments the infected eggs were incubated at 39°C. which is recommended for the best development of the chick. After many unsuccessful attempts were made to get any visible reactions on the membranes, a study was made to determine if the temperature of incubation was a factor in the viability of the virus on the membrane.

Twenty ten-day embryos were divided into four groups of five. Four embryos were inoculated with 0.1 cc. of 1:20 dilution of tissue extract and one served as a control. The groups were incubated at the following temperatures: 33°C., 35°C., 37°C., and 39°C. No definite changes were visible in any of the membranes of the 20 embryos at the end of 36 hours. But at the end of 48 hours, in all the membranes incubated at 37°C. there appeared small grayish white focal thickenings from 0.1 to 0.5 mm. in size, and some of them took the form of white glistening elevated streaks about 0.3 mm. in width and 3 mm. or more in length. At the same time three of the four embryos incubated at 39°C., and two of those incubated at 35°C. showed very small grayish thickenings, while those incubated at 33°C. and the controls were negative.

The changes in the membranes incubated at 37°C. progressed steadily in size and extent up to 96 hours, while those incubated at 39°C. showed little progress after 72 hours. Those at 33°C. showed little change at the end of 96 hours and the controls were negative.

At the end of 5 days all membranes were harvested and inocu-

lated into rabbits. The animals which were inoculated with membranes incubated at 33°C., 35°C., and 37°C. died on the 8th day, and those receiving 39° virus, died at the end of 10 days with typical symptoms of myxomatosis. This indicated that enough virus to kill an animal remained viable on the membranes regardless of the incubation temperature for at least 5 days.

Serial passages. Hence the question now arose as to whether the changes brought about were due to the amount of virus and the length of time for which it remained viable on the membrane or if there was an actual multiplication of the virus during incubation. A serial passage was then attempted.

In as much as the membranes incubated at 37°C. showed earlier and more profuse lesions than those incubated at 33°C., 35°C. and 39°C., this was chosen for the temperature for incubation.

At this time it was thought essential to determine the minimum amount of tissue containing the infecting agent necessary to produce characteristic lesions, and also to see the effect of varying methods of inoculation on the appearance and the size of the lesion. Twelve 10-day embryos were inoculated with 0.1 cc. of dilutions of myxomatous rabbit tissue varying decimally from 10^{-1} to 10^{-5}. Two embryos were used for each dilution, one of which was previously prepared by making a small slit of about 0.2 mm. in length in an avascular area of the membrane with the point of a sterile scalpel to allow the virus inoculum to come in contact with the cells of all three germ layers directly. All the embryos were incubated at 37°C. and examined after 96 hours for typical gross changes. The results expressed in table 1 show that a dilution of 10^{-2} was the highest dilution producing any changes in the injured membrane and 10^{-3} the highest in the uninjured membrane. The tumor tissue was active in the rabbit in 10^{-5} dilution, hence it takes roughly one hundred times as much material to get a reaction on the membrane as it takes in the rabbit (table 1).

Trauma caused by puncture with an exposure of the underlying germ layers to the virus directly does not further the development of the changes. This was further confirmed by attempted

injection of the inoculum directly into the allantoic cavity by means of a fine needle and by planting small pieces of bacteria-free myxomatosis tissues on the membranes of the embryo directly.

A dozen fertile eggs were incubated for each serial passage, and 6 to 8 were inoculated with 0.1 cc. of a 1:20 dilution of myxomatous tissue as was described above. Two controls were used in each series. All were incubated for 96 hours at 37°C. At the end of that time three of the embryos showing the most profuse lesions on the membrane were selected, aseptically harvested and transferred to sterile Petri dishes. Adherent moisture was carefully removed by rolling each membrane on to sterile filter paper until blotting was complete. All membranes were tested for

TABLE 1

Minimum infective dose of virus on chorio-allantoic membrane

DILUTION OF VIRUS	MEMBRANE PUNCTURED	MEMBRANE INTACT
10^{-1}	++++	+++
10^{-2}	++	+++
10^{-3}	0	+
10^{-4}	0	0
10^{-5}	0	0
Controls	0	0

++++, numerous lesions; +, few lesions.

bacterial contamination by inoculating 0.5 gram in glucose infusion broth. After the three membranes were weighed, sterile physiological salt solution was added to make a 1:10 tissue dilution and the tissue was triturated with sterile ground Pyrex; the suspension was transferred to a sterile centrifuge tube and centrifuged at 700 revolutions for five minutes. The controls were similarly treated. The supernatants were used as reinocula for the next passage, 0.1 cc. of the 1:10 membrane dilution being employed in each case. In all the 400 embryos studied only, 2 or 3 membranes were found bacterially contaminated.

At each passage the virus content of the inoculum was titered on a rabbit. Decimal dilutions from 10^{-1} to 10^{-6}, were prepared and two intradermal injections of 0.1 cc. of each dilution were

made 3 cm. apart on the animal's shaven flank. Readings were taken on the 5th day after injection and a raised inflammatory

TABLE 2

Titer of the virus myxomatosis obtained in thirty-three serial passages on chorio-allantoic membrane of chick embryo

SERIES NUMBER	HIGHEST INFECTIVE DILUTION	READING OF DOUBLE INTRADERMAL INOCULATION ON RABBITS	
1	10^{-3}	+	+
2	10^{-4}	+	−
3	10^{-4}	+	−
4	10^{-4}	+	−
5	10^{-3}	+	+
6	10^{-4}	+	+
7	10^{-4}	+	+
8	10^{-4}	+	+
9	10^{-3}	+	+
10	10^{-4}	+	+
11	10^{-4}	+	+
12	10^{-5}	+	−
13	10^{-3}	+	+
14	10^{-3}	+	+
15	10^{-2}	+	+
16	10^{-2}	+	+
17	10^{-4}	+	−
18	10^{-4}	+	−
19	10^{-3}	+	−
20	10^{-4}	+	+
21	10^{-4}	+	−
22	10^{-3}	+	−
23	10^{-4}	+	−
24	10^{-4}	+	+
25	10^{-4}	+	+
26	10^{-5}	+	+
27	10^{-5}	+	−
28	10^{-4}	+	+
29	10^{-6}	+	+
30	10^{-4}	+	+
31	10^{-6}	+	−
32	10^{-5}	+	+
33	10^{-5}	+	+

area of 3 mm. at the point of injection was considered a positive reaction.

The virus as indicated in table 2 was thus carried in serial trans-

fer through 33 passages on the chorio-allantoic membrane. The infective titer of the membranes may be seen to vary between 10^{-2} and 10^{-6} in the membranes selected for titration in the various series, tending toward an average between 10^{-3} and 10^{-4}. In only one case did the titer drop as low as 10^{-2}, in four series it was as high as 10^{-5}, and in two attained a maximum of 10^{-6}. The concentration of the virus in the membrane tissue was in most cases less than that in myxomatous rabbit tissue, although at times it approximated the latter. In the last seven passages the average titer of the virus seemed definitely higher than in previous passages, a fact which may indicate a gradually increasing pathogenicity for the chick membranes. There seemed considerable individual variation in susceptibility among chick embryos as some membranes develop only a small number of lesions, while others become heavily infected when inoculated with the identical amount of virus suspension.

It does not seem possible that dilution contained in the original infective dose could be responsible for the viability of the virus at the end of the 33rd passage (fig. 7). The original inoculum contained 5000 animal infecting doses; if one assumes there is no multiplication but merely dilution of the virus, at the end of the 3rd transfer, estimating the dilution upon the basis of membrane weight of original infecting dose, about one sixth of a dose would remain. The fourth passage shows a titer for rabbits in a tissue dilution of 1:1000 to be 10^{-4}. This seems to point to an actual multiplication of the virus in the membrane.

RELATION OF CHILLING THE EMBRYOS TO THE PRODUCTION OF LESIONS

During the course of the experiment it was noted that embryos which had been accidentally chilled by exposure to a temperature of 25°C. for 12 to 18 hours before or after inoculation developed more profuse changes in the membranes than those which were not chilled. Four groups of 8 10-day embryos each were used to confirm this observation. Each group of 8 was subdivided, 4 of the embryos being chilled at 25°C. for 12 hours before inoculation and 4 kept at incubation temperature throughout

the experiment, 37°C. The 4 groups of 8 were then inoculated with 0.1 cc. of a 1:10 dilution of an infected membrane from the 28th series and incubated at 33°C., 35°C., 37°C., and 39°C. for 96 hours. Membranes in each group were pooled, weighed and extracts from each group titrated intradermally on rabbits.

The results read in terms of the reactions on rabbits as described in table 1 showed the greatest apparent difference at 33°C. where the titer was a hundred times greater in the chilled than in the unchilled membranes, and a titer higher than any previously attained. The chilling showed little effect on those incubated at 35°C. and 37°C., but seemed to reduce the titer at 39°C. As stated before, infected membranes incubated at 33°C.

TABLE 3

Titers obtained in virus grown in chilled and unchilled membranes at varying temperatures

TEMPERATURE	CHILLED BEFORE INOCULATION			UNCHILLED		
	Number of eggs	Titer	Reaction on rabbits	Number of eggs	Titer	Reaction on rabbits
°C.						
33	4	10^{-6}	++	4	10^{-4}	++
35	4	10^{-5}	+−	4	10^{-4}	+−
37	4	10^{-4}	++	4	10^{-4}	+−
39	4	10^{-1}	++	4	10^{-3}	++

and 35°C. showed little or no change, but when they were chilled they showed lesions which were less discreet and elevated than those produced at 37°C., but contained as much or more virus than the latter.

CHANGES BROUGHT ABOUT BY THE VIRUS IN THE MEMBRANES

From the above results the virus apparently had multiplied on the membrane. Therefore a study of the membrane both macroscopically and microscopically was made to determine if these changes were specific. No lesion was observed on the membrane until after 36 hours of incubation when small grayish white focal thickenings were apparent (fig. 1) which at the end of 48 hours (fig. 2) appeared as glistening areas of 0.2 to 1 mm.

in length having a distinct elevation. During this period they were discreet and scattered patches which by the end of 84 hours (fig. 5) had a definite tendency toward elongation and extension along definite lines. All membranes did not respond the same to equivalent inoculums, some showing an interlacing net work of lesions over the entire inoculated area (fig. 6), while others were less abundant and more discrete (fig. 5) and still others more confluent (figs. 3 and 4).

The linear character of the lesion was its most conspicuous feature. In smaller lesions it was associated with the presence of a capillary. This was later confirmed by microscopic examination of infected membranes (fig. 12). In larger lesions the capillaries were not always present. Danchakoff (1917) has shown that between the 10th and 15th day of embryonic development of a chick the position of the capillary respiratory network in the allantois undergoes a change. It first lies entirely beneath the ectodermal layer, but capillary sprouts during that period grow up into and through the ectoderm until the capillary network ultimately comes to the surface. It is highly probably that this phenomenon is related to the development of the lesions and that such lesions consisting principally of ectodermal proliferation, occur under the stimulation of the virus at points where capillaries have penetrated into the ectoderm and supply abundant nourishment to adjacent ectodermal cells.

Examination of microscopic sections of infected membranes revealed changes common to those reported by workers on other viruses. The most significant change was found in the ectodermal layer. Starting as early as at the end of 12 hours there appeared small areas of ectodermal proliferation with slight mesodermal edema (fig. 10). By thirty-six hours there was an infiltration of polymorphonuclear leucocytes in both ectoderm and mesoderm. The entoderm was not involved until the end of 48 hours, when there was a noticeable proliferation of this layer in the neighborhood of the proliferating ectoderm (fig. 11). These changes continued to progress until at 96 hours all layers were heavily involved, the mesodermal edema was pronounced, and the membrane had increased in thickness fourfold (fig. 14). At

this time, or before in some of the large ectodermal lesions, necrosis was evident with the formation of a cap of necrotic material (fig. 13).

D'Aunoy and Evans (1937) claimed to have found such changes in normal aging membranes of chick embryos. It was also noted that certain non-specific lesions occurred; although controls used for sections were inoculated with heat-inactivated virus suspension, in no case was there found in the sections examined from control membranes lesions resembling those described above.

A study of Giemsa-stained tissue spreads from infected and normal membranes was made in order that these might be compared with similar spreads from infected rabbit tissue. One of the most striking features of the latter is the presence of numerous large stellate, or myxoma cells containing large, very granular nuclei, and at times exhibiting certain characteristic granules within their cytoplasm. Such cells have not been observed in spreads from virus-infected membranes, although occasionally somewhat hypertrophied mesodermal cells containing within their cytoplasm clusters of granular bodies were found.

The granular eosinophilic inclusions first described by Rivers (1926–7) and occurring in the cytoplasm of epithelial cells overlying tumorous areas in infected rabbits, have not yet been found in spreads from infected membranes.

There have been noted consistently however in such spreads granular bodies staining a purplish red with Giemsa stain and varying in size from barely resolvable particles to the size of a large coccus. Frequently bipolar or dumbbell shaped forms occur. These bodies have been found in the cytoplasm of ectodermal, mesodermal and occasionally entodermal cells. They also occur in large mononucler phagocytes and in polymorphonuclears. They seem similar to granules occurring in the cytoplasm of certain of the myxoma cells and in hypertrophied monocytes and macrophages in the rabbit tissue both because of their staining qualities and their variation in size and shape.

Hobbs (1928) has suggested that such granules in the cytoplasm of myxoma cells may represent the virus itself.

SUMMARY AND CONCLUSIONS

The virus, *Myxomatosum*, produced a change on the chorio-allantoic membrane of the developing chick embryo. The form and abundance of this lesion was influenced by the size of the infective dose as well as by the chilling of the embryos before and after inoculation and by the temperature at which the infected membranes were incubated. There was no correlation between the profuseness of lesion on the membrane and the titer of the virus obtained from it. The fact that the virus passed through 33 serial passages over a period of 132 days without evident change in its character seems to bring the viability at the end of that time beyond the possibilities of dilution into one of actual multiplication of the causative agent.

The lesions formed by the virus were distinguished by their linear character which seems to be associated with the presence of capillaries in the ectoderm. The changes in the ectoderm were similar to those found in the case of other viruses grown on chorio-allantoic membranes of chick embryos and consist principally of ectodermal proliferation.

A pathological change apparently similar to that found in myxomatous tissue of rabbits was the occurrence of granular bodies in the cytoplasm of ectodermal, mesodermal and entodermal cells of the membrane.

From the above facts it may be concluded that the chorio-allantoic membrane of the chick embryo may be used as a medium for the study of the growth of the virus *Myxomatosum.*

REFERENCES

BENJAMIN, BERNARD, AND RIVERS, F. M. 1931 Regeneration of virus myxomatosum in presence of cells of exudates surviving in vitro. Proc. Soc. Exp. Biol. and Med., 28, 791–792.

DANCHAKOFF, V. 1917 The position of the respiratory vascular net in the allantois of the chick. Amer. Jour. Anat., 21, 407–420.

D'AUNOY, RIGNEY, AND EVANS, FLORENCE L. 1937 The histology of the normal chorio-allantoic membrane of the developing chick embryo. Jour. Path. and Bact., 44, No. 2, 369–377.

HOBBS, J. R. 1928 Studies on the nature of infectious myxoma virus of rabbits. Amer. Jour. Hyg., 8, 800–839.

HYDE, ROSCOE R., AND GARDNER, RAYMOND E. 1933 Infectious myxoma of rabbits. Amer. Jour. Hyg., 17, 446–465.

LEWIS, MARGARET REED, AND GARDNER, RAYMOND E. 1932 A simple method for studying the cytology of infectious myxoma of the rabbit. Amer. Jour. Path., 8, 583–587.

RIVERS, F. M. 1926–27 Changes observed in epidermal cells covering myxomatous masses induced by virus myxomatosum (Somarelli). Proc. Soc. Exp. Biol. and Med., 24, 435–437.

WOODRUFF, ALICE MILES, AND GOODPASTURE, ERNEST W. 1931 The susceptibility of the chorio-allantoic membrane of chick embryos to infection with the fowl pox virus. Amer. Jour. Path., 7, 209–222.

After the above paper had gone to press, there came to our attention in the Australian Journal of Experimental Biology and Medical Science, 1937, 15: 2, 131–139, a paper by Dora Lush which confirms in part our findings.

PLATE I

FIGS. 1–6. Successive stages found at 36, 48, 60, 72, 84 and 96 hours on the chorio-allantoic membranes of chick embryos infected with virus myxomatosum.

FIG. 7. Seventy-two hour control membrane inoculated with inactivated virus suspension.

FIG. 8. Infected membrane at the 33rd serial transfer.

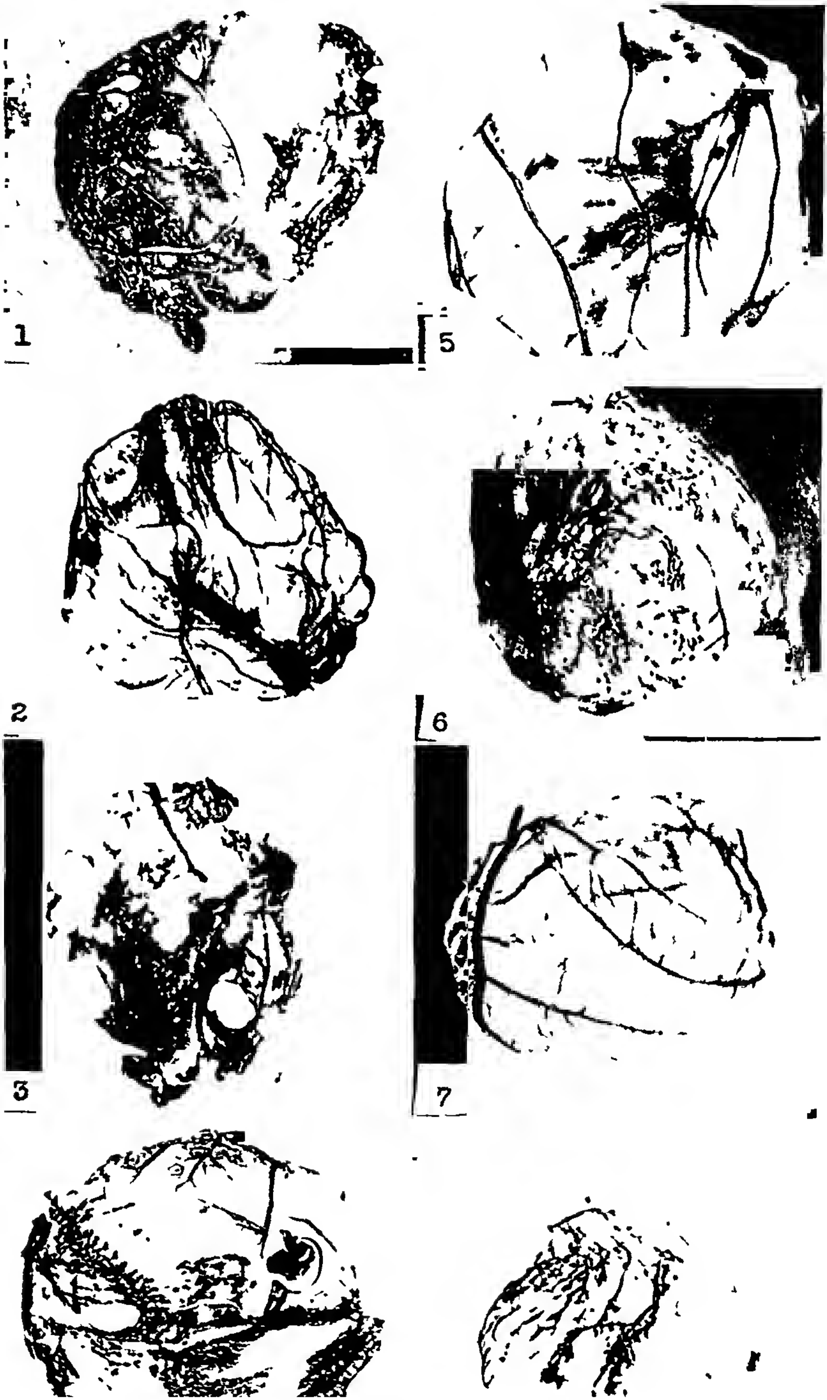
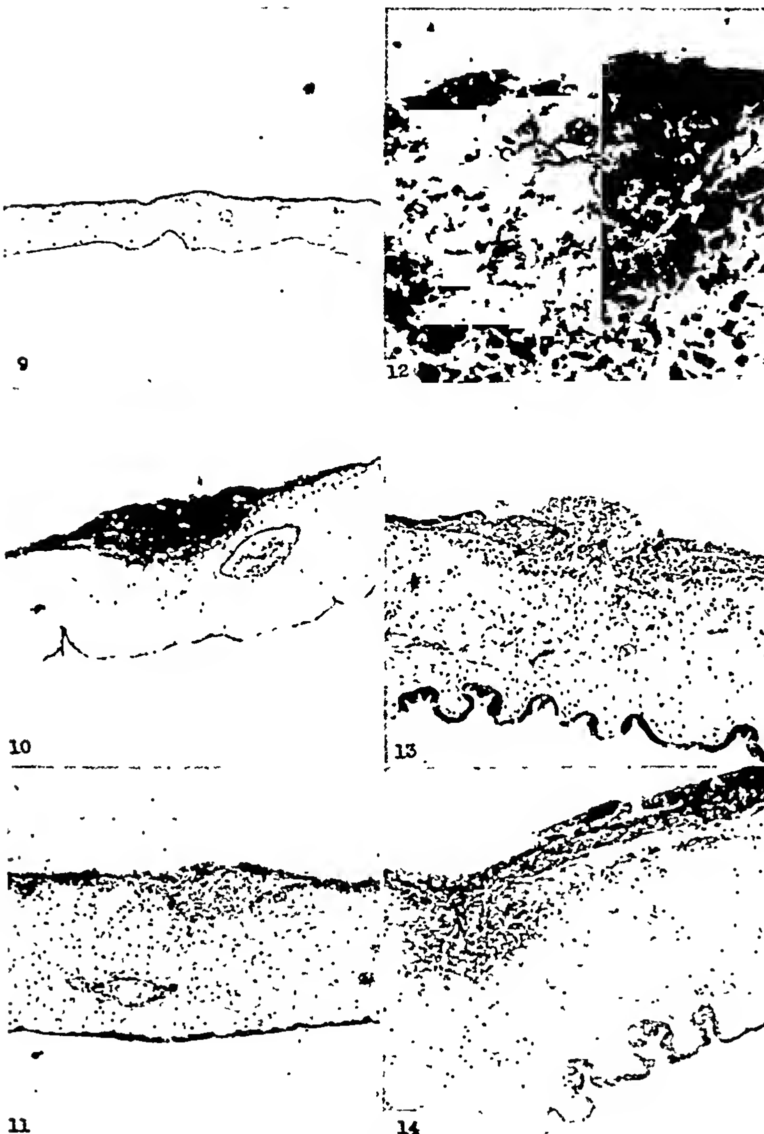

1
5
2
6
3
7

PLATE II

Fig. 9. Eighty-four hour control membrane inoculated with heat inactivated rus.

Figs. 10–14. Sections of infected membranes at 12, 48, 72, and 96 hours of cubation. ×100.

Fig. 12. 500 × magnification of ectodermal patch shown in Figure 11.



(Rachel E. Hoffstadt and K. Stephen Pilcher: Study of Virus Myxomatosum)

THE EFFECT OF SODIUM CHLORIDE ON THE Eh OF PROTOGENOUS MEDIA

L. S. STUART[1] AND LAWRENCE H. JAMES[2]

Bureau of Chemistry and Soils, United States Department of Agriculture

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A number of investigators (Lefevre and Round, 1919; Stather and Liebscher, 1929, a and b; Stuart, Frey and James, 1933) have shown that those bacteria capable of growing on media containing high concentrations of sodium chloride prefer alkaline conditions for growth. Specifically it has been reported (Stather and Liebscher, 1929 b and Stuart, Frey and James, 1933) that the optimum pH for the growth of these bacteria was 8, although it has been observed that this optimum may vary for any isolated organism from 7 to 9 depending upon the concentration of sodium chloride in the substrate.

Although the foregoing observations can be said to be essentially true, there is some doubt as to the significance of pH in expressing the electrolytic condition of a sodium chloride solution wherein the Na and Cl ions undoubtedly exceed the number of H and OH ions. In the electrometric determination of pH in a solution of this character the error due to the effect of the Na ion probably would be as great or greater than the actual H ion concentration itself.

Since the Eh value is a direct measure of the total electrometric energy it probably describes more accurately than pH the comparative electrometric conditions existing in low and high salt media. In ordinary laboratory media the Eh value, in general, decreases with increasing pH values and the work of recent

[1] Associate Bacteriologist.

[2] Formerly Senior Bacteriologist, Bureau of Chemistry and Soils, United States Department of Agriculture, now Department of Bacteriology and Animal Pathology, University of Maryland, College Park, Maryland.

investigators (Hewitt, 1936) indicates that such values are fully as important as pH in their effects upon bacterial growth.

It would seem possible then that the optimum high pH previously observed might be associated with a relatively low Eh value, and the apparent changes in the pH values optimum for growth in the presence of varying concentrations of salt might be due to changes in the Eh values rather than to actual H-ion concentration changes. A study was therefore made to determine the effects of sodium chloride in low and high concentrations upon the Eh of certain laboratory media.

A large volume of liquid medium was made up containing 1 per cent proteose-peptone, 2 per cent bacto-gelatin and 200 cc. of yeast water per liter. This was than divided into 12 equal volumes and these adjusted by the addition of N/1 HCl or N/1 NaOH to pH 3.93; 4.66; 4.85; 4.91; 5.37; 5.64; 5.74; 6.14; 6.42; 7.04; 7.37; and 8.0, respectively, using the quinhydrone electrode and measuring the pH after sterilization.

Each of these adjusted volumes was then subdivided into 12 volumes to which C.P. sodium chloride (Baker's analysed) was added to give the following concentrations: 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, and 27.5 per cent. This provided 144 lots of media and each lot was tubed separately in cotton-plugged sterile tubes and sterilized in the autoclave at 15 pounds steam pressure for 20 minutes. They were removed from the autoclave, cooled to 30°C. and Eh measurements made on duplicate tubes.

Eh was determined using a potentiometer with a 3.5 N KCl reference half-cell and a platinum electrode. All readings were made at 28° to 30°C. in the presence of flowing nitrogen and are expressed in terms of the reference half-cell.

Although occasional drifts were encountered, the readings as a whole were found to be remarkably constant after the nitrogen had been bubbled through the sample for a few minutes. This seemed to hold true regardless of NaCl concentration.

The results are recorded in table 1.

In figure 1 representative Eh values are plotted against initial pH of the media at different sodium chloride concentrations.

TABLE 1

Eh of sterilized peptone-gelatin-yeast water media of various pH values and salt concentrations

pH*	PER CENT SODIUM CHLORIDE											
	0	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.5
	mv.	*mv.*	*mv.*	*mv.*	*mv.*	*mv.*	*mv.*	*mv.*	*mv.*	*mv.*	*mv.*	*mv.*
3.93	319	312	313	329	324	304	289	290	324	290	309	290
4.66	323	316	272	282	289	277	299	272	262	256	286	261
4.85	317	311	275	277	271	271	279	278	327	266	282	276
4.91	310	314	292	279	323	298	275	268	269	275	270	258
5.37	282	279	292	282	279	268	258	243	243	241	240	241
5.64	293	296	298	277	277	241	243	295	286	236	287	239
5.74	268	266	266	263	266	265	252	228	242	229	234	234
6.14	266	260	268	259	263	260	230	216	220	221	225	228
6.42	249	253	268	268	239	244	220	205	206	220	213	226
7.04	233	230	238	236	228	230	235	203	193	198	199	195
7.37	237	224	222	232	222	222	201	200	196	192	197	194
8.00	214	211	211	230	215	214	183	193	192	192	191	188

* Quinhydrone determination on salt-free sterilized media.

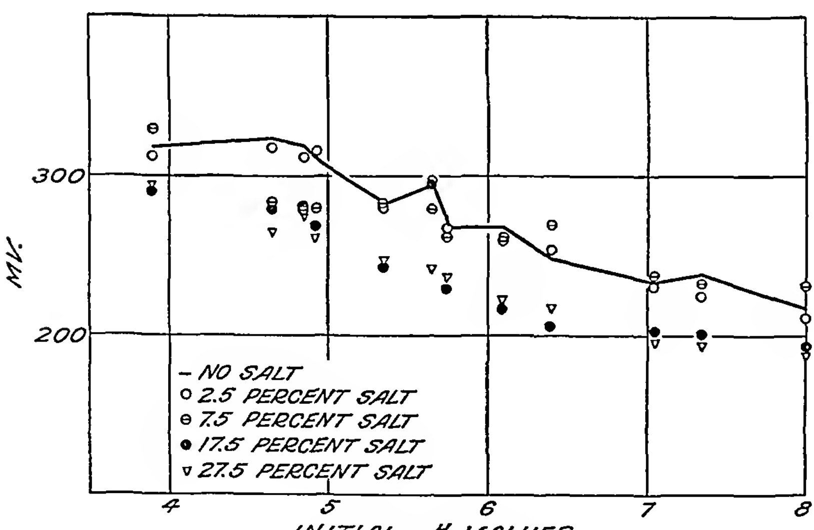

FIG. 1. CHANGES IN Eh WITH INCREASING pH IN A PROTOGENOUS MEDIUM TO WHICH VARIOUS CONCENTRATIONS OF SALT WERE ADDED

In general, a higher original pH is accompanied by a lower Eh value regardless of the amount of salt present. In the absence of added salt the decrease is reasonably uniform but, when salt is added, variations in the trend appear, particularly in the media having low original pH values.

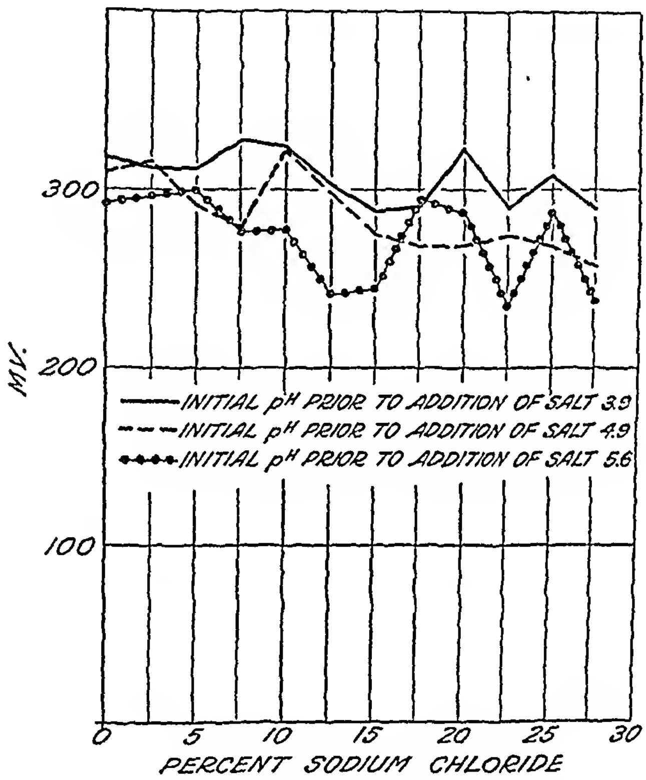

Fig. 2. Effect of Increasing Concentrations of Sodium Chloride on Eh of Media Having Initial pH Values of 5.6 or Lower

In table 1 it can be observed that the greatest irregularities secured are between pH 4.66 and 5.74. In view of the fact that this range of pH values probably includes the isoelectric points of the various protogenous constituents of the medium, such variations might be expected.

In figures 2 and 3 some of the Eh values shown in table 1 are plotted against salt concentration.

General decreases in Eh, but with wide fluctuations with increasing concentrations of added NaCl, are shown in those media having original pH values of 5.64 and below (fig. 2). In those media with original pH values of 5.74 and above (fig. 3), the fluctuations are not so marked, but there are significant abrupt decreases at 15 and 17.5 per cent NaCl. Above 17.5 per cent changes in Eh in the increasing salt concentrations are not great.

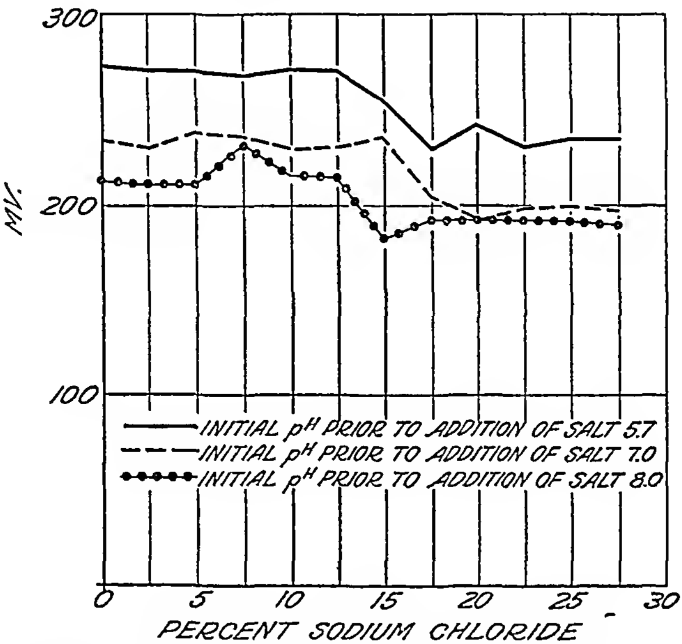

FIG. 3. EFFECT OF INCREASING CONCENTRATIONS OF SODIUM CHLORIDE ON Eh OF MEDIA HAVING INITIAL pH VALUES OF 5.7 OR HIGHER

Concentrations of 17.5 per cent salt or higher are usually employed in media for the cultivation of obligate halophiles. From these results it seems reasonable to assume that favorable conditions previously designated by high pH values would have been more significantly designated as relatively low Eh values.

The studies just described were made with a sterilized medium containing peptone, gelatin and yeast water so that it was not

known in what measure the observed changes in Eh could be attributed to the addition of NaCl alone, to sterilization, or to the action of the various ingredients upon one another. In order to observe, in a measure, some of these influences individually the Eh of solutions of the constituents of the basic medium with various quantities of added salt was determined both before and after steam sterilization at 15 pounds for 20 minutes. All measurements were made as previously described.

TABLE 2

Eh in millivolts before and after sterilization of unadjusted peptone, gelatin and yeast water solutions containing increasing concentrations of sodium chloride

SOLUTION		PER CENT SODIUM CHLORIDE									
		0	2.5	5	7.5	10	12.5	15	17.5	20	22.5
		mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.
1 per cent proteose-peptone; 6.57*	Unsterilized	175	175	175	192	191	174	126	145	129	169
	Sterilized	174	165	174	168	178	167	167	169	166	172
	Difference	−1	−10	−1	−24	−13	−7	+41	+24	+37	+13
2 per cent bacto-gelatin; pH 6.43*	Unsterilized	182	178	178	200	205	183	159	167	165	173
	Sterilized	170	192	200	179	198	205	211	298	257	227
	Difference†	−12	+14	+22	−21	−7	+22	+52	+131	+92	+54
200 cc. yeast water per liter; pH 6.16*	Unsterilized	200	200	200	212	215	202	199	200	196	202
	Sterilized	181	200	210	211	207	208	212	208	202	200
	Difference	−19	0	+10	−1	−8	+6	+13	+8	+6	−2

* Quinhydrone determination.

† Plus sign indicates increase and minus sign decrease in millivolts after sterilization.

Separate solutions of 2 per cent bacto-gelatin, 1 per cent proteose-peptone and 200 cc. of yeast water per liter were made in boiled distilled water. The pH of these solutions, as determined with the quinhydrone electrode, were 6.43, 6.57 and 6.16, respectively. Each solution was then divided into 10 equal aliquots, to one of which no salt was added. To the remaining nine, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 and 22.5 per cent C.P. NaCl were added, respectively. They were allowed to stand in a cold room (45°F.) over night. Eh measurements were made on por-

tions of the solutions the next day. The remainder of each aliquot was then sterilized at 15 pounds steam pressure for 20 minutes, brought to 30°C. and Eh measurements again made. The results are shown in table 2.

These results show that the addition of salt to gelatin, peptone, and yeast water solutions without sterilization has practically no effect on the Eh value of these solutions when the concentration of salt does not exceed 5 per cent. With 7.5 and 10 per

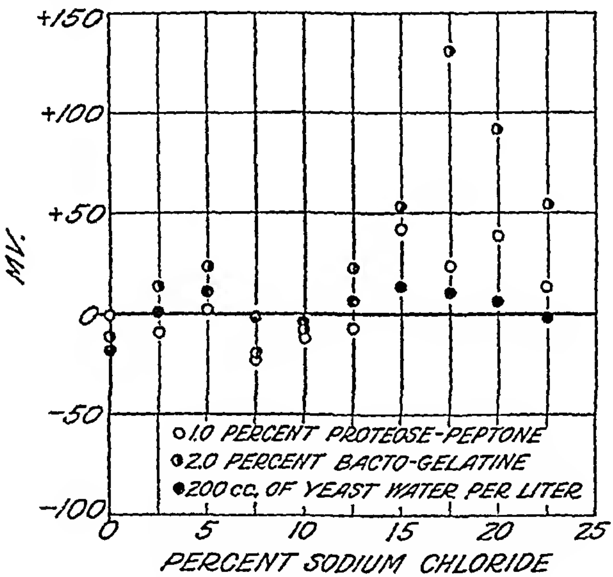

FIG. 4. CHANGES IN Eh OF MEDIA INGREDIENTS DUE TO STERILIZATION AT 15 POUNDS FOR 20 MINUTES

cent salt the Eh value is slightly increased for all three solutions but with the higher concentrations marked decreases in Eh were found in both the gelatin and peptone solutions. In the yeast water solution the decrease with higher concentrations of salt was not so great but the Eh values were considerably lower than those for solutions containing 7.5 and 10 per cent of salt.

After sterilization, marked increases in Eh were observed with the gelatin and peptone solutions of high salt content, and slight increases for the yeast water solutions of similar salt concentra-

tions. Slight decreases in Eh were found for all of the solutions with the intermediary concentrations of 7.5 and 10 per cent. In the presence of 5 per cent salt a slight increase in Eh was found in all solutions. With 2.5 per cent salt the results show an increase in Eh for the gelatin, a decrease for the peptone and no change for the yeast water. In the absence of salt there was a slight decrease in Eh. These results show the marked influence of heat sterilization upon the Eh value of a culture medium, particularly when large amounts of salt are present. These changes in Eh with sterilization are shown diagrammatically in figure 4.

Inasmuch as the effect of sterilization in high concentrations of salt (as shown in table 2 and fig. 4) might be interpreted as a heat oxidation catalysed by salt, an additional study was made *to determine the effect of added increasing* concentrations of salt upon the poise of solutions of the media constituents.

Thirty cc. aliquots of the sterilized 2 per cent solutions of bacto-gelatin and salt reported in table 2 were pipetted into glass stoppered 50 cc. cylinders and 0.1 cc. of 3 per cent H_2O_2 added, shaken thoroughly, and allowed to stand for one hour. Eh measurements were then made. In making these particular readings it required about five minutes using flowing nitrogen to eliminate potential drifts. The results are recorded in table 3 and presented diagrammatically in figure 5.

These results are particularly interesting since they show a definite increase in the poise in the gelatin solution to which salt has been added. In general the smallest effects of the oxidant were found in the higher concentrations of salt. This may have been due to increasing concentrations of salt stabilizing the peroxide or buffering the medium itself against oxidations.

The greater oxidation in the lower salt concentrations is the reverse of the results obtained in the effects of sterilization on Eh change, where the greater oxidations were found in the media of high salt content. It is suggested that in solutions of such nutrients as were tested there are a certain number of oxidizable groups present. During sterilization in high salt media a portion of these are oxidized. Thus, after sterilization a portion of these

TABLE 3

Effect on Eh of adding H_2O_2 to sterilized gelatin-salt solutions

NaCl	2 PER CENT GELATIN SOLUTION		INCREASE IN Eh
	Before addition of H_2O_2	After addition of H_2O_2	
per cent	*mv.*	*mv.*	*mv.*
0	170	377	+207
2.5	192	367	+175
5.0	200	355	+155
7.5	179	363	+184
10.0	198	356	+158
12.5	205	353	+148
15.0	211	352	+141
17.5	298	347	+49
20.0	257	349	+92
22.5	227	343	+116

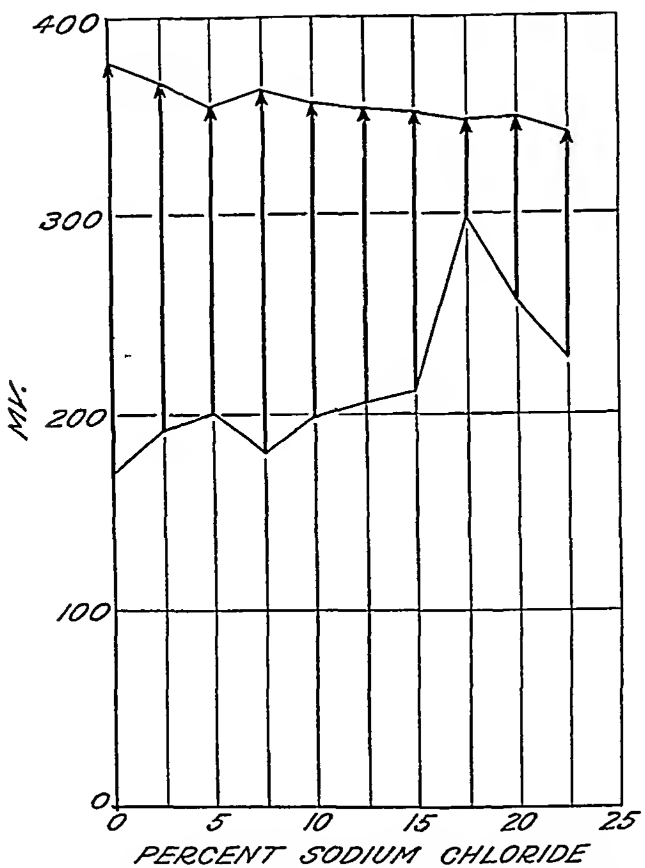

FIG. 5. INCREASE IN Eh OF GELATIN-SALT SOLUTIONS DUE TO THE ADDITION OF HYDROGEN PEROXIDE

groups have not been oxidized, the larger proportions remaining in the solutions containing the lower concentrations of salt. Some of these remaining groups can be oxidized, however, by an active oxidant such as H_2O_2. In this connection it may be noted (fig. 5) that the smallest oxidation brought about by the H_2O_2 was in a solution in which by far the greatest oxidation had already taken place, not necessarily in the presence of the greatest amount of salt; and, that in general the amount of oxidation induced by the H_2O_2 depended upon the amount that had already taken place during sterilization. Nevertheless, the extent of

TABLE 4

Effect on Eh of adding cysteine hydrochloride to sterilized peptone salt solutions

NaCl	BEFORE ADDITION OF CYSTEINE HCl	AFTER ADDITION OF CYSTEINE HCl	DECREASE IN Eh
per cent	*mv.*	*mv.*	*mv.*
0	174	118	−56
2.5	165	129	−36
5.0	174	136	−40
7.5	168	137	−31
10.0	178	140	−44
12.5	167	138	−29
15.0	167	134	−33
17.5	169	134	−35
20.0	166	140	−26
22.5	172	137	−35

the total oxidation of the system was progressively less with increasing concentrations of added salt. Thus, sodium chloride in high concentrations may catalyze an oxidation in the sterilization of media to a certain potential at which it may then stabilize it somewhat against further oxidation. From the determinations made originally on the sterilized media it is apparent that with such heterogeneous substrates the amount of oxidation in the high salt media due to heat is not as great as the amount of reduction due to the combined action of increasing pH and salt content.

Another test of this stabilizing or poising effect was made by

adding a uniform amount of reductant (cysteine hydrochloride) to sterilized proteose-peptone solutions containing increasing quantities of NaCl. One cubic centimeter of a .02 per cent solution of the reductant was added to 15 cc. aliquots of the sterilized 1 per cent proteose-peptone solutions referred to in table 2, shaken and allowed to stand one hour when Eh measurements were again made. The results are shown in table 4, and figure 6.

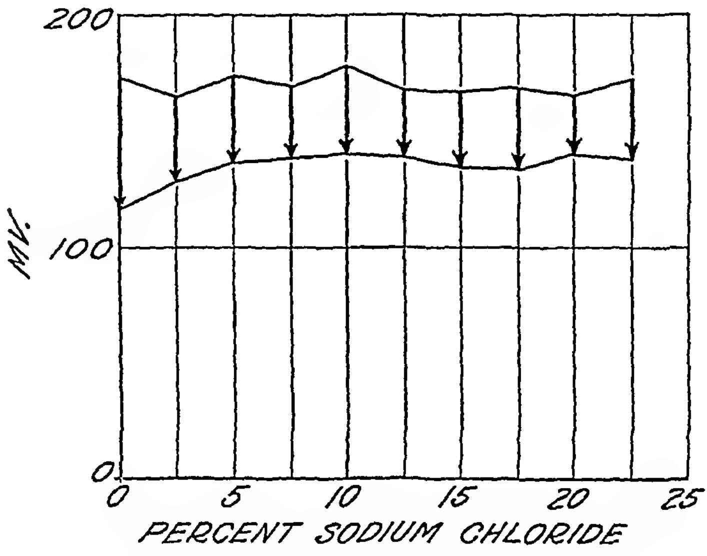

FIG. 6. DECREASES IN Eh OF PEPTONE-SALT SOLUTIONS DUE TO THE ADDITION OF CYSTEINE HYDROCHLORIDE

These figures show that even small amounts of added salt may interfere with reduction by cysteine hydrochloride although this effect does not appear to be markedly affected by further increases in the concentration of sodium chloride. The results furnish additional evidence that the addition of sodium chloride increases the poise of a medium.

DISCUSSION AND SUMMARY

The results of these studies suggest the following generalizations.

The Eh of protogenous media decreases with increases in both initial pH and the amount of sodium chloride added prior to sterilization.

The presence of sodium chloride in protogenous media adds to the poise. As the concentration of sodium chloride increases, the medium may become more firmly poised against oxidation, although the extent of change induced by an active oxidant such as H_2O_2 at any given concentration depends primarily on the initial potential of the protogenous-salt mixture.

The presence of sodium chloride intensifies the poise against the action of cysteine hydrochloride.

Protogenous media containing high concentrations of sodium chloride may be materially oxidized during sterilization.

In view of what is known (Hewitt 1936) regarding variations between even different lots of media of supposedly the same general composition it is probable that with different lots of peptone and gelatin, and with other proteins or protein derivatives, there would be wide deviations from the specific results reported, although such deviations should not be great enough to alter the basic effects reported herein.

REFERENCES

HEWITT, L. F. 1936 Oxidation-reduction Potentials in Bacteriology and Biochemistry. 4th ed. London County Council.

LEFEVRE, E., AND ROUND, L. A. 1919 A preliminary report upon some halophilic bacteria. Jour. Bact., 4, 177–182.

STATHER, F., AND LIEBSCHER, E. 1929a Ueber das rotwerden gesalzener rohhäute (3. mitteilung über häuteschäden von M. Bergmann und F. Stather). Collegium, No. 713: 437–450.

STATHER, F., AND LIEBSCHER, E. 1929b Zur Bakteriologie des rotwerdens gesalzener rohhäute (4. Mitteilung über häute und lederschäden von M. Bergmann und F. Stather). Collegium, No. 713, pp. 437–450.

STUART, L. S., FREY, R. W., AND JAMES, LAWRENCE H. 1933 Microbiological studies of salt in relation to the reddening of salted hides. U. S. D. A. Tech. Bul. 383.

THE EFFECT OF Eh AND SODIUM CHLORIDE CONCENTRATION ON THE PHYSIOLOGY OF HALOPHILIC BACTERIA

L. S. STUART[1] AND LAWRENCE H. JAMES[2]

Bureau of Chemistry and Soils, United States Department of Agriculture

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Numerous investigators (Brown, 1922; Clayton and Gibbs, 1927; Clarke, 1923; Robertson, 1931; Smythe, Whitney and Zo Bell, 1937) have observed and reported obligate halophilism for bacterial cultures. Although it is not the intention of this paper to contend that such bacteria do not exist it seems only fitting to draw attention to the fact that none of the so-called obligate halophiles examined by the authors have failed to produce growths of some kind on media containing very little or no added salt (sodium chloride) when cultures 30 days old or older have been used for inoculation purposes. Obligate halophilic effects have been observed with transfers from young rapidly growing cultures, but these same cultures when allowed to age for 30 days or longer have been found to be capable of growth upon transfer to media of low salt content. When the type of organic nutrient in the medium is varied, these growths are often entirely different in appearance from the mother culture, so much so as to be unrecognizable, either through loss of pigmentation, changes in cellular morphology or in the type of macroscopic growth in liquid or on solid substrates. Because of these changes in the appearances of growths they are and rightly should be regarded with considerable skepticism, since it is always possible that they may have been due to dormant contaminants.

[1] Associate Bacteriologist.

[2] Formerly Senior Bacteriologist, Bureau of Chemistry and Soils, United States Department of Agriculture, now Department of Bacteriology and Animal Pathology, University of Maryland, College Park, Maryland.

However, in a previous report (Stuart, 1935) it was shown that the organisms causing the flesh reddening of salted hides, although having a highly variable morphology depending upon the organic constituents of the medium, were affected only as to size by varying concentrations of sodium chloride. By using a medium of constant organic composition and making repeated microscopic examinations it now seems possible to determine with a reasonable degree of accuracy whether growths are directly related to the inoculum or are the result of contamination.

The present study was made to determine the effect of Eh and varying concentrations of sodium chloride on the growth and physiological activity of a typical red chromogenic halophile. A medium containing 2.0 per cent bacto-gelatin, 1.0 per cent proteose-peptone and 200 cc. of yeast water per liter was made up with 12 different percentages of salt varying from no salt at all to 27.5 per cent. To aliquots of each of these lots of media N/1 NaOH and N/1 HCl were added to produce a wide range of Eh values. Duplicate tubes of media of each Eh value were then inoculated with *Sarcina littoralis* (Paulsen) from a 60-day growth on a salt saturated agar medium as follows: A loop needle transfer of the growth was suspended in 100 cc. of sterilized saturated NaCl solution and 0.1 cc. was then added to each tube of medium. These, with duplicate control sterile tubes, were placed in the incubator at 37.5°C.

Since it has been observed by Lochhead (1934) and others that bacteria in media of high salt content grow better on the surface of solid media than in liquid broths, a strip of filter paper was placed in each tube of these media before sterilization to provide a surface effect comparable to that found with a solid substrate. Where surface growths are indicated in the subsequent studies they do not refer to pellicle formation but to growth at the junction of the filter paper and the surface of the liquid substrate. The strips of white filter paper also provided an excellent background for the detection of pigment development.

Eh measurements were made on tubes of sterilized media from each lot at the beginning of the incubation period using a 3.5 N KCl reference half-cell and a platinum electrode with flowing

nitrogen and working at 28 to 30°C. All Eh values are reported in terms of the reference half-cell.

The time at which growths first appeared and the type of growth in each tube were recorded. After 30 days incubation all tubes showing growths and all control tubes were brought back to volume with boiled distilled water and samples removed for Eh determinations. Growths were carefully examined for the following properties: (1) Pigment production; red-yellow or absence of; (2) growth distribution, diffuse or surface; (3) microscopic appearance of cells.

The results of these observations are briefly summarized in table 1.

The data recorded in table 1 shows some highly interesting correlations.

A zone of comparatively rapid growth is shown in those media containing no salt, and 2.5, 5.0, 7.5 and 10.0 per cent salt respectively. Growths occurred in media having initial Eh values of 310 and 312 millivolts with no salt and 2.5 per cent of salt respectively, of 298 millivolts with 5.0 per cent salt, of 279 millivolts with 7.5 per cent salt, and of 266 millivolts with 10 per cent salt. As judged by the development or lack of growth it appears that a greater tolerance for a medium of high Eh is shown by this organism at low salt concentrations. In this zone, growth was uniformly diffused throughout the medium and non-pigmented, with the exception of growth in one set of the series of media containing 7.5 per cent salt having an initial Eh of 279 millivolts. In all the media mentioned above the microscopic appearance of the cells was not changed except that a considerable reduction in size was observed over those in the parent culture.

With 12.5 per cent salt, growth was scanty and flocculated. The cocci were highly irregular in size. Growth was considerably delayed and no pigment was produced. With this concentration of salt the highest initial Eh value at which growth occurred was 265 mv.

With 15.0 per cent salt, pigmented growths similar to those of the parent culture occurred in those media having initial Eh values of 201 and 183 millivolts. In this same medium at 235

millivolts a scanty flocculated growth similar to that found in 12.5 per cent salt and producing no pigment was found. No growths were found in media of this concentraton of salt having initial Eh values higher than 235 mv.

TABLE 1

Effect of initial Eh and salt concentration of media on growth of Sarcina littoralis (Paulsen), and changes in Eh of media due to bacterial growth

NaCl	Range of initial Eh values covered	Highest initial Eh value at which growth occurred	Range covering changes in Eh in inoculated media after correction for changes in controls smallest to greatest	Lag periods prior to growth	Type of growth	Pigment	Microscopic appearance of cells
per cent	*mv.*	*mv.*	*mv.*	*days*			
0	214–323	310	−5 to −89	1–3	Diffuse	None	Sarcina
2.5	211–316	312	−33 to −94	1–2	Diffuse	None	Sarcina
5.0	211–313	298	−14 to −61	1–7	Diffuse	None	Sarcina
7.5	230–329	277*	−15 to −62*	1–10	Diffuse*	None*	Sarcina
10.0	215–324	266	−25 to −53	1–10	Diffuse	None	Sarcina
12.5	214–304	265	−21 to −52	14–21	Flocculate	None	Irregular cocci
15.0	183–299	235	−115 to +38†	14–21	Flocculate and surface	White† and pink	Irregular cocci
17.5	183–295	203	+17 to +37	14–21	Surface	Red	Irregular cocci
20.0	192–327	220	+32 to +47	10	Surface	Red‡	Sarcina
22.5	192–290	229	+15 to +76	7–14	Surface	Red	Sarcina
25.0	191–309	229	+5 to +67	7–10	Surface	Red	Sarcina
27.5	188–290	228	+19 to +47	10	Surface	Red	Sarcina

* Growth occurred also at 279 mv. but was surface in character producing an orange pigment.

† Growth on media at 235 mv. non-pigmented while growths at 201 mv. and below were pigmented.

‡ Growth in media at 192 mv. non-pigmented.

In media containing 17.5 per cent salt growths did not occur with initial Eh values above 203 millivolts. Growths were typically surface in character and produced a red pigment.

In media containing 20 per cent sodium chloride growth occurred at 220 millivolts, with 22.5 and 25.0 per cent at 229 millivolts, and with 27.5 per cent at 228 millivolts. All growths in

these media were typically surface in character and the cells were identical with those of the inoculum. With the exception of one lot of media having an initial Eh value of 192 millivolts with 20 per cent salt, all growths were brilliantly pigmented and the intensity of the red pigment appeared to increase with growths in those media having lower Eh values.

These results show a decreased tolerance of high initial Eh values with increasing concentrations of salt up to 17.5 per cent.

TABLE 2

Changes in Eh of sterilized medium during incubation at 37.5°C. for 30 days regardless of salt content

ORIGINAL Eh OF MEDIUM	NUMBER OF MEASUREMENTS	AVERAGE INCREASE OR DECREASE IN Eh
mv.		*mv.*
300 or over	3	−16
290–300	5	−11.8
280–290	1	−7
270–280	6	+27.3
260–270	12	+35.2
250–260	2	+74.5
240–250	3	+62.3
230–240	8	+97.5
220–230	13	+88.8
210–220	5	+126.2
200–210	4	+106.8
190–200	11	+127.3
180–190	3	+142.5

With concentrations higher than 17.5 per cent growths occur at considerably higher initial Eh values but tolerance for high initial Eh values is not as great as was found with cultures growing in 15 per cent of salt or less.

Before considering the changes in the Eh values of the culture media due to bacterial growth, some mention should be made of the changes in the Eh of sterile control tubes during the incubation period. In table 2 the average increases or decreases in Eh for those lots of uninoculated media having initial values within a 10 millivolt range and covering a total range from 180 to 300 millivolts and above are presented. In making these

averages no attention was given to salt concentrations. These figures show that the greatest changes occur in those tubes having low initial Eh values. That is, in general, the more reduced the medium the greater the changes due to auto-oxidation and the more oxidized the initial medium the smaller the changes during incubation.

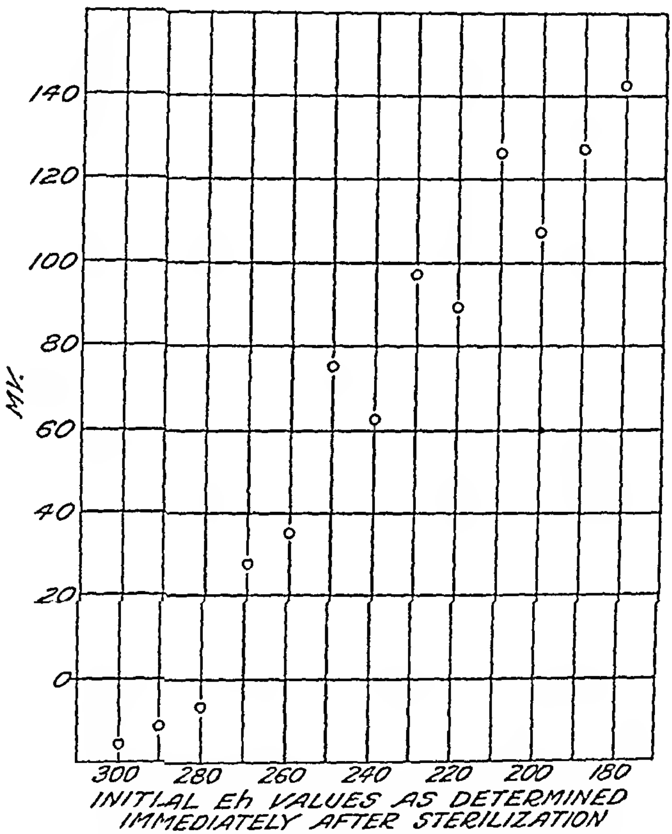

FIG. 1. INCREASES IN Eh OF STERILIZED MEDIA WITH VARIOUS INITIAL Eh VALUES AFTER 30 DAYS INCUBATION AT 37.5°C.

These changes are presented diagrammatically in figure 1. In general they are similar to those previously found (Stuart and James, 1938) by the use of H_2O_2, and are in accord with the findings of other investigators (Hewitt, 1936).

In table 3 are presented the average increases in Eh during

TABLE 3

Effect of salt concentration on increases in Eh of sterilized media during incubation at 37.5°C.

NaCl	RANGE OF Eh VALUES PRIOR TO INCUBATION	NUMBER OF MEASUREMENTS	AVERAGE INCREASE IN Eh
per cent	*mv.*		*mv.*
0	220–240	2	+102.0
2.5	220–240	2	+105.5
5.0	220–240	2	+84.0
7.5	220–240	3	+107.7
10.0	220–240	3	+106.0
12.5	220–240	3	+90.7
15.0	220–240	1	+92.0
20.0	220–240	2	+90.5
22.5	220–240	2	+72.5
25.0	220–240	3	+73.0
27.5	220–240	2	+75.0

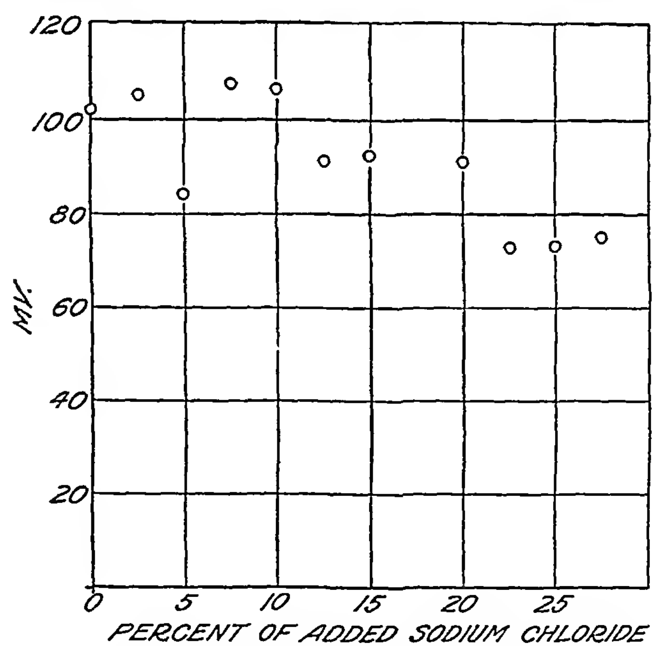

FIG. 2. DECREASING AMOUNTS OF AUTO-OXIDATION IN STERILIZED MEDIA WITH INCREASING AMOUNTS OF ADDED SODIUM CHLORIDE

incubation for those lots of sterile media having initial Eh values falling between 220 and 240 millivolts for each salt concentration employed. Here it can be seen that with relatively uniform initial Eh values the extent of auto-oxidation decreases with increasing concentrations of salt. This also agrees with previous findings (Stuart and James, 1938) in which H_2O_2 was used as an oxidant. These results are presented diagrammatically in figure 2.

From tables 2 and 3 and figures 1 and 2 it is apparent that the changes in the sterile controls must be taken into consideration in the interpretation of the results obtained in those media supporting bacterial growth. Since measurements were not made periodically for either the controls or the cultures in these studies it is assumed that the deviation from the incubated sterile controls represents the sum total of change due to bacterial growth, although this in some instances involves the interpretation of potentials higher than those found in the original medium as reductions.

In figures 3 and 4 typical changes for representative lots of media at each salt concentration in which growth occurred are presented diagrammatically. In these figures the change of potential in the incubated sterile control is first shown followed by the deviation from the control due to bacterial activity.

From these figures it can be observed that, in those media containing 12.5 per cent salt or less where growth occurred, the Eh was lower than with the incubated control sterile medium, with one exception. This exception was in the medium containing 7.5 per cent salt with an initial Eh of 279 millivolts. No change was observed in the sterile control for this particular lot of medium and the growth that occurred produced a bright orange pigment. The tubes in which growth occurred had a decidedly higher Eh than the control.

In general, the effect of the growths in all the low salt media (with the exception previously mentioned) was a reduction.

With those media containing 15.0 per cent salt, 2 lots showed marked increases in Eh and 1 lot a marked decrease due to bacterial action. With the lots showing an increase (table 1) red

pigment was produced, whereas no pigment was produced in that lot of medium that showed a decrease in Eh.

With 17.5 per cent salt and above, where red pigments were produced, the growth of the bacteria was accompanied by a

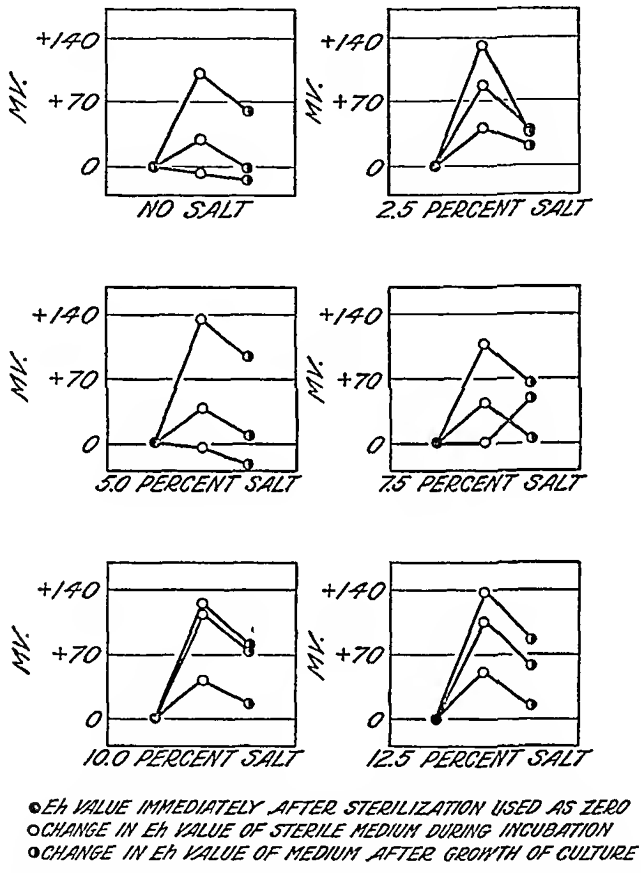

FIG. 3. TYPICAL CHANGES IN INDIVIDUAL LOTS OF STERILIZED MEDIA DUE TO AUTO-OXIDATION AND BACTERIAL GROWTH AS SHOWN IN TABLE 1

definite increase in Eh. In the one case where pigment was not produced which has been previously referred to, a marked decrease in Eh was found. These results showed that the production of pigment by *Sarcina littoralis* (Paulsen) is accompanied by an oxidative type of metabolism.

Since it has been observed that the amount of oxidation by H_2O_2 and the changes in the sterile controls were governed by the initial Eh, an attempt was made to determine from the results in table 1 whether the amount of oxidation attributable to bac-

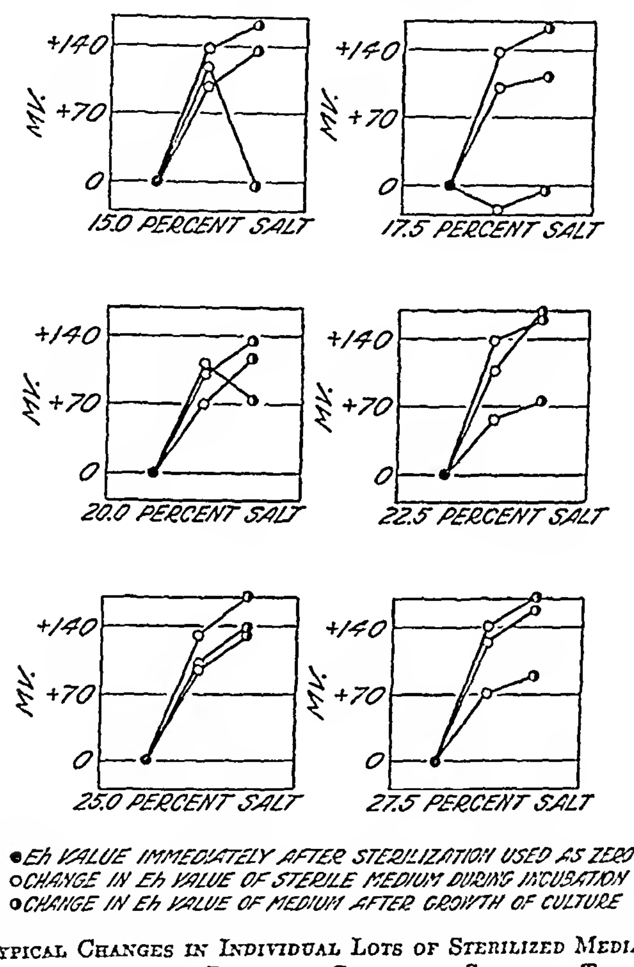

FIG. 4. TYPICAL CHANGES IN INDIVIDUAL LOTS OF STERILIZED MEDIA DUE TO AUTO-OXIDATION AND BACTERIAL GROWTH AS SHOWN IN TABLE 1

terial growth was also governed by the initial Eh value. Those tubes of media showing oxidations due to bacterial action were placed in five groups according to the Eh values of the incubated controls, as follows: 300 millivolts and below; 300 to 310 mil-

livolts; 310 to 320 millivolts; 320 to 330 millivolts; and 330 millivolts and above. The average potential for each group of controls was determined, as was the average increase in potential

TABLE 4

Effect of initial Eh of media on increase in Eh due to growth of chromogenic bacteria*

NUMBER OF MEASUREMENTS	RANGE OF INITIAL Eh VALUES	AVERAGE INCREASE IN Eh DUE TO BACTERIAL ACTION
	mv.	*mv.*
6	285–300	+42.2
6	300–310	+45.6
6	310–320	+37.3
5	320–330	+32.0
2	330–350	+12.0

* Based on incubated controls.

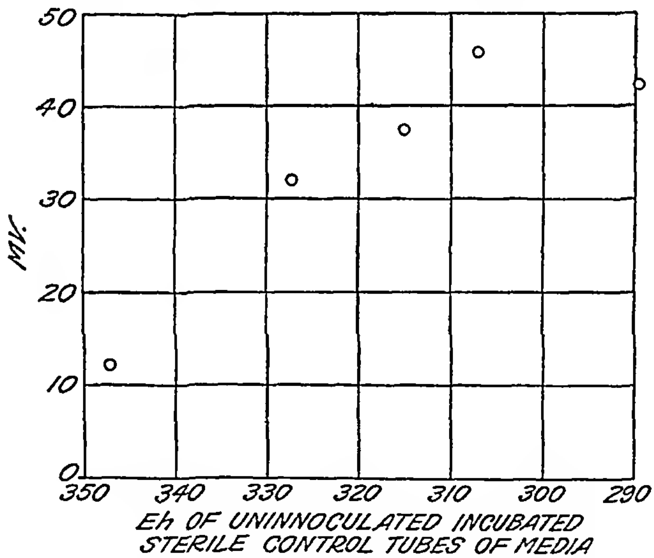

FIG. 5. EXTENT OF INCREASES IN Eh DUE TO GROWTH OF CHROMOGENIC BACTERIA AS INFLUENCED BY THE INITIAL Eh OF THE MEDIUM

due to bacterial growth. The results are recorded in table 4 and presented diagrammatically in figure 5. These show definitely that the amount of oxidation due to bacterial action tends to

TABLE 5

Effect of initial Eh on extent of reduction by non-chromogenic bacteria regardless of NaCl concentration*

RANGE OF INITIAL Eh VALUES	NUMBER OF MEASUREMENTS	AVERAGE DECREASE IN Eh DUE TO GROWTH OF BACTERIA
mv.		*mv.*
280 or under	3	−13.7
280–290	7	−17.6
290–300	5	−40.6
300–310	8	−30.2
310–320	4	−33.2
320–330	3	−26.6
330–340	9	−47.3
340–350	5	−46.0
350 and above	2	−73.0

* Based on incubated controls.

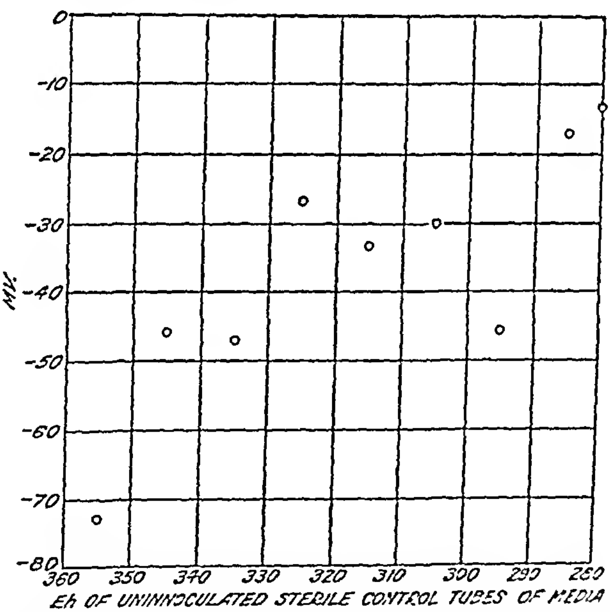

FIG. 6. EXTENT OF DECREASES IN Eh DUE TO THE GROWTH OF NON-CHROMOGENIC BACTERIA AS INFLUENCED BY THE INITIAL Eh OF THE MEDIUM

increase with the decrease in the Eh of the substrate, similar to the variation in oxidation by H_2O_2 and the oxygen of the air.

Similar figures compiled from the tubes showing a reduction

due to bacterial activity show in general a greater reduction with media of high initial Eh than for media of low initial Eh. This is indicated in table 5 and figure 6.

DISCUSSION

The inhibiting and retarding effect on the growth of *Sarcina littoralis* (Paulsen) of intermediary concentrations of salt as shown in table 1 is presented diagrammatically in figure 7. The results

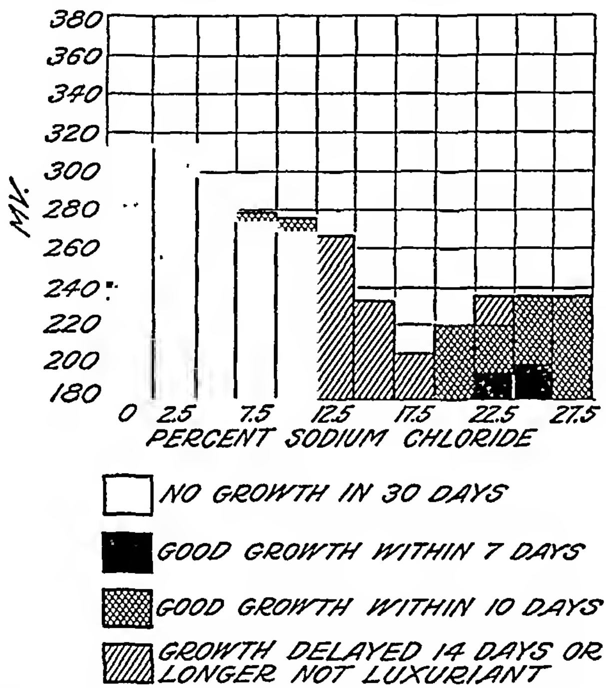

Fig. 7. Growth of Sarcina Littoralis Paulsen on Media of Increasing Concentrations of Salt at Different Initial Eh Levels

would seem to be highly significant since with bacteria isolated and propagated on media of low salt content it is often impossible to "step up" rapidly growing vegetative cultures through these concentrations even when the same culture will grow on media containing higher percentages of salt. These intermediary concentrations are likewise inhibitory when attempting to acclimate

a culture isolated on a high salt medium to media of low salt content by making transfers from rapidly growing cultures through a series of media of diminishing concentrations of salt. These intermediary percentages of sodium chloride, where growth was retarded, correspond closely to those concentrations of salt causing the greatest depression in the molal freezing point curve, hence they have the highest osmotic pressures and lowest ionic activity coefficients. They also correspond closely to that part of the surface tension curve where with increasing concentrations of salt there is little or no increase or decrease in surface tension. Thus, there probably are definite reasons why growth is inhibited at these concentrations.

In general the tolerance for high initial Eh values observed for the different concentrations of sodium chloride employed would appear to be governed primarily by either osmotic pressure or ionic activity. Since Eh is actually a measure of ionic activity the latter factor is probably fully as important as the former. Further studies will be necessary to determine the actual effect of the different physical factors involved.

The finding of an oxidative type of metabolism when pigment was produced and a reductive type of metabolism in the absence of pigment production strongly suggest that the pigment may play a definite rôle in the oxidation although it may be that it constitutes a reversible oxidation-reduction system (Hewitt, 1936) and in this case is merely acting as an indicator of the final potential in the substrate. However, in the media of low salt content where no pigment was produced the final potentials were in many instances as high or higher than the final potentials obtained with the pigmented cultures on media of high salt content. Hence, the former possibility seems the more probable.

SUMMARY

Sarcina littoralis (Paulsen) did not show obligate halophilic characteristics when transferred from old cultures.

In media containing 10 per cent added salt or less transfers from sixty (60) day cultures of this organism grew rapidly without the production of pigment.

Growth in these media was uniformly diffused and occurred at higher Eh values as the concentration of salt decreased.

These non-pigmented growths caused a decrease in the Eh of the medium over the corresponding uninoculated incubated sterile controls.

Growth was delayed in those media containing 12.5 and 15 per cent of salt longer than in media containing either higher or lower concentrations of salt.

Growth in media containing 17.5 per cent salt or more was not diffused throughout the substrate but occurred only at the junction of the filter paper with the surface of the liquid. It was pigmented.

Low Eh values were more favorable for growth in media containing 17.5 per cent salt or more.

The red pigmented growths attached to the filter paper strips increased the Eh values of the media.

The extent of change in Eh in the sterilized incubated controls increased with lowered initial Eh values.

The amount of change in Eh in the sterilized incubated controls decreased as the concentration of salt increased.

Increases in the Eh of high salt media due to the growth of the chromogenic bacteria were greater in media having lower initial Eh values.

Decreases in the Eh of low salt media due to the non-chromogenic growth of bacteria were in general greater in those media having the higher initial Eh values.

REFERENCES

Brown, W. W. 1922 Halophilic bacteria. Soc. Exp. Biol. and Med. Proc., **19**, 321–322.

Clayton, Wm., and Gibbs, W. E. 1927 Examination for halpohilic microorganisms. Analyst, **52**, 395–397.

Clarke, P. C. 1923 Red discolouration (so-called "pink" or "pink eye") on dried salted fish. (Gt. Brit.) Dept. Sci. and Indus. Research Food Invest. Bd. Spec. Rpt. 18, 23 p.

Hewitt, L. F. 1936 Oxidation-Reduction Potentials in Bacteriology and Biochemistry, 4th Ed., London County Council.

Lochhead, A. G. 1934 Bacteriological studies on the red discoloration of salted hides. Canadian Jour. Res. **10**, 275–86.

Robertson, M. E. 1931 A note on the cause of certain red colorations on salted hides and a comparison of the growth and survival of halophilic or salt-loving organisms and some ordinary organisms of dirt and putrefaction on media of varying salt concentrations. Jour. Hyg. (London), 31, 84–95.

Smythe, W. Whitney, and Zo Bell, Claude 1937 Direct microscopic evidence of an indigenous bacterial flora in Great Salt Lake. Jour. Bact., 32, 1, 75.

Stuart, L. S. 1935 The morphology of bacteria causing reddening of salted hides. Jour. A. L. C. A., 30, 5, 226–235.

Stuart, L. S., and James, Lawrence H. 1938 The effect of sodium chloride on the Eh of protogenous media. Jour. Bact., 35, 369–380.

THE STRICT ANAEROBES IN THE SLIME AND INTESTINES OF THE HADDOCK (GADUS AEGLEFINUS)

JAMES M. SHEWAN

Torry Research Station, Aberdeen, Scotland

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Numerous surveys have been made of the bacterial content of the slime and intestines of freshly caught marine fish in the course of the study of their spoilage by microörganisms during transport to the market and storage by various methods. A previous paper from this Station (Stewart, 1932) presented the results of a systematic survey of the aerobic organisms of the slime and intestines of the haddock (*Gadus aeglefinus*). The present paper records an attempt to gain some information on the nature of the strict anaerobes of the same materials.

Little information on the strict anaerobes of the slime and intestines of freshly-caught fish is available. Fellers (1926) found none on the slime of freshly caught salmon, although members of the group *Clostridium* were present in the spoiled fish. Hunter (1920), on the other hand, found no strict anaerobes in decomposing salmon. Reed and Spence (1929) always found various species of *Clostridia* in the intestines of haddock, but gave no details of the types isolated; nor did they mention either the presence or absence of anaerobes in the slime.

Obst (1919), who studied the swelling of canned "sardines" (herring, *Clupea harengus*), isolated two obligate, gas-forming anaerobes from the gills and intestines of fresh herring, and from the schizopods and copepods on which they fed. From the descriptions given it is impossible to identify them. Schönberg (1930) found the anaerobe, *Bacillus putrificus* (Bienstock), on fish (fresh-water and marine), but from his description it is apparent that he was dealing with decomposing fish. Previous

work, therefore, has done little more than to hint at the presence of anaerobes in the intestines of fresh fish and their absence from the slime.

TECHNIQUE AND METHODS

Haddock, caught by line a few miles off Aberdeen were placed, with a minimum of handling, in a sterile container. The laboratory examination was started within 3 to 6 hours.

Samples of the slime were removed with a platinum loop or by rubbing the surface of the fish with a sterile cotton-wool swab and were spread on agar or inoculated into nutrient broth.

Samples of the intestinal contents (upper and lower) were obtained by placing the fish in a glass box, previously sprayed to remove air-borne organisms, and making an incision along the middle line of the ventral surface with a sterile scalpel. The samples were placed either in broth or spread directly on agar. In a few cases portions of the faeces were also examined. For convenience all these results have been grouped together as of "intestinal contents."

The media used for the isolation of the organisms and their subsequent purification were nutrient agar and broth made from horse's heart. The Wilson (1928) modification of the McIntosh and Fildes metal jar was used. The jars were evacuated with a "Hyvac" pump, hydrogen introduced and the electric current passed for about 1 hour, and were then incubated at 20° or at 37°C. The same types of organisms occurred at both temperatures, with quicker growth at 37°C., and subsequent work was therefore done at the higher temperature. Care was taken to ensure the purity of the cultures studied, and the presence of many facultative anaerobes rendered this task long and laborious. After the isolation of pure cultures, sub-cultures on agar slopes were set up, both aerobically and anaerobically, and only the obligate anaerobes were further investigated.

The biochemical properties of the types were tested in the usual way. For the fermentative tests, the following substances were used: glucose, lactose, sucrose, mannitol, dulcitol, arabinose, xylose, laevulose, galactose, maltose, raffinose, starch, inulin,

dextrin, glycerol, adonitol, sorbitol, and salicin. The indol, Voges-Proskauer, (acetyl-methyl-carbinol), sulphuretted-hydrogen and nitrate-reduction tests were made in the usual way (Topley and Wilson, 1931), while the action on coagulated serum, coagulated white of egg, cooked-meat medium, gelatine and litmus-milk was also noted. The cultures were incubated at least 14 days before the anaerobic jar was opened and the biochemical tests made.

The morphology was determined from agar slopes and broth, usually after 3 and 10 to 14 days. Sometimes blood-agar was used, since certain of the types spored best thereon. Tests for motility were also made.

The similarity of the morphological and biochemical properties of many of the non-pathogenic anaerobes has rendered their exact naming difficult, especially since many essential data are lacking in Bergey's *Manual of Determinative Bacteriology* and elsewhere. Information as to the antigenic constitution of these organisms, upon which classification might be established, is also almost completely lacking (see however Kimura 1924, McCoy and McClung 1936, etc.). A study of this problem is in progress in the Bacteriology Department, Marischal College, Aberdeen.

No attempt was determined to assess the exact frequency with which a particular type occurred, but some general conclusions on this subject are given later.

THE SLIME

Numerous attempts were made to isolate strict anaerobes from the slime of fresh haddock. In no instance were they found, and the conclusion seems justified that they are absent. Fellers, as mentioned, reached the same conclusion with salmon.

THE INTESTINE

On the other hand, strict anaerobes were always encountered in the haddock's intestine, sometimes in considerable numbers. The cultures belong to the genus *Clostridium*, and fall into two groups, one with round spores, the other with oval spores.

Group A. Rods with round spores

Type I. Morphology: Young (3 days) cultures on agar show long, slender rods with rounded ends in pallisade groups; the length varies, but is usually 10 to 12μ. In older cultures the organism shows a tangled mass of long filaments like actinomyces, but with no branching which is quite characteristic. Frequently spiral and bulging forms are present. The spores are strictly terminal, very like those of *Clostridium tetani*, but not quite so large, and are never present in great numbers, even in old cultures. Spores appear most readily on blood-agar. The organism is actively motile with peritrichous flagella; is Gram-positive in young cultures, but soon becomes Gram-negative on ageing. No capsule is present.

Colonies are irregular, effuse, transparent and rounded, with a fine filamentous edge. The most characteristic feature is the relatively slow growth, visible colonies appearing only after 3 to 5 days. The colonies are dull and oily in appearance. On ageing they appear to grow into the agar, and acquire a ground-glass aspect.

In broth (sucrose or nutrient) and in peptone water, growth is always poor, even after 14 days; turbidity is slight and there is no sediment or growth at the surface.

Biochemical reactions

Glucose: Acid and gas.
Glycerol: Acid and gas?
Sucrose: Acid and gas?
After 4 weeks.
Indol: Negative.
Voges-Proskauer: Negative.
Nitrate reduction: Negative.
H_2S: Positive.
Gelatin liquefaction: Positive.
Litmus milk: Peptonised.
Coagulated sheep serum
Coagulated egg albumin
Slowly digested and liquefied.
Cooked meat medium: Little digestion, slight or no blackening.

Catalase: Negative.
Peroxidase: Negative.
Haemolysis: Weakly positive.
Fluorescence on MacConkey's medium: Negative or weakly positive.

The organism is non-pathogenic, and appears to be related to *Clostridium lentoputrescens* of Hartsell and Rettger (1934) and *Clostridium putrificum* of Morgan and Wright (1934), with the

latter of which it was compared under the same experimental conditions. It has been shown, however, to be distinct serologically from the organism of Morgan and Wright, obtained from the National Collection of Type Cultures, London. Without entering into the controversy on the question of the identity of *Clostridium putrificus* Bienstock (see Cunningham (1932), Reddish and Rettger (1922, 1923, 1924), Morgan and Wright (1934), Weinberg and Ginsbourg (1927)), it is concluded that this organism is of the *C. putrificum* type.

Type II. On agar, young cultures are slender, Gram-positive rods, 3 to 4μ long, arranged singly and never in filaments. The spores are round, terminal, and, when first formed, stain solidly in a manner typical of the tetanus group. They are readily formed in most media (cf. type I), but are particularly abundant on blood-agar and broth. The organism is motile, and has no capsule.

Single colonies have not been obtained, since the organism always spreads over the plate in a thin, effuse, dull, translucent film. The spreading edge is finely filamentous, and, in colour, whitish yellow. In fluid media growth is only fair, with uniform turbidity, little sediment and a putrid odour.

Biochemical reactions

Laevulose: Acid and gas?
Indol: Negative.
Voges-Proskauer: Negative.
Nitrate reduction: Negative
H_2S: Negative.
Gelatin liquefaction: Positive.
Litmus milk: Slight clot and alkaline.
Coagulated sheep serum: Softened, but no liquefaction or blackening.
Coagulated egg albumin: Poor growth, no digestion.
Cooked meat medium: Little digestion or blackening, good growth otherwise.
Catalase: Negative.
Peroxidase: Negative.
Haemolysis: Positive.
Fluorescence on MacConkey's medium: Negative.

The organism is pathogenic, causing tetanus in experimental animals, and serologically belongs to the type 5 of the tetanus group.

Type III. Young cultures on agar are composed of slender rods, 4 to 6μ long, with rounded ends, occurring singly or in pairs, with a few longer forms. The spores, which are round and

terminal, are very like those of the tetanus group but not so large. They are easily formed on a medium of cooked-meat, but with difficulty on blood-agar (cf. tetanus, which spores abundantly on blood-agar). The bacilli are actively motile.

Single colonies, which are easily obtained, are irregular, round, with a diameter of 4 to 6 mm., finely pitted and coarsely lobate. The centre is slightly raised, and the colony is whitish, opaque, and granular in appearance. On blood-agar the colonies are more effuse and furrowed, and have a shining lustre.

In broth, as in most fluid media, growth is only fair, with uniform turbidity, a slight granular sediment, and a foetid odour.

Biochemical reactions

Glucose: Acid and gas?
Indol: Strongly positive.
Voges-Proskauer: Negative.
Nitrate reduction: Negative.
H_2S: Negative.
Gelatin liquefaction: Negative.
Litmus milk: Unchanged.
Coagulated sheep serum: Not liquefied but somewhat softened.
Coagulated egg albumin: Unchanged, growth poor, thin and effuse.
Cooked meat medium: Digestion, with some blackening and putrid smell.

Catalase: Negative.
Peroxidase: Negative.
Haemolysis: Negative.
Fluorescence on MacConkey's medium: Positive.

The organism is non-pathogenic, and from its cultural and morphological features appears to be related to *Bacillus tetanoides A.*, (Adamson, 1918). Tests of agglutination showed it to be distinct from the strain of *Clostridium tetanomorphum* of the National Collection of Type Cultures, London.

Group B. Rods with oval spores

Type I. On agar this organism appears as short, stout rods with square ends, occurring usually singly or in pairs side by side, and less frequently in chains of 10 or so. Some longer forms are always present. The spores are central or subterminal, and when first formed do not swell the rod. The organism is motile and Gram-positive in young cultures.

After 3 days' incubation on agar, the colonies are small and round; the centre is raised; the margin appears granular and pitted; and the edge is regular, but later becomes serrulate.

The colour is whitish and the consistency viscid, the whole colony tending to be lifted off when handled with the platinum needle.

In contrast to the 3 types of group A, growth is excellent in fluid media, giving a thick, mucous sediment and a very putrid smell.

Biochemical reactions

Glucose: Acid and gas.
Maltose: Acid and gas.
Glycerol: Acid and gas.
Adonitol: Acid and gas.
Sucrose: Acid and gas?
Laevulose: Acid and gas?
Inulin: Acid and gas?
Indol: Negative.
Voges-Proskauer: Negative.
Nitrate reduction: Negative.
H_2S: Positive.
Gelatin liquefaction: Positive.
Litmus milk: Peptonised and darkened.
Coagulated sheep serum: Completely liquefied in a few days.
Coagulated egg albumin: No apparent change.
Cooked meat medium: No apparent change.
Catalase: Negative.
Peroxidase: Negative.
Haemolysis: Positive.
Fluorescence on MacConkey's medium: Positive.

The organism is non-pathogenic, and may be related to *Clostridium bifermentans*.

Type II. Young cultures on agar are composed of slender bacilli, 6 to 8μ long, with rounded ends, usually in pairs end to end; in broth they frequently occur in clumps or palisade groups. Some long forms may be present. The spores are large, oval, terminal or sub-terminal, swell the rod, and appear to be readily formed on all media. The organism is actively motile and is Gram-positive (later Gram-negative). On agar the colonies are characteristic. They are opaque to translucent, and grow down into the medium; the shape is irregular and roundish; the surface is slightly raised and finely pitted; the colour is yellowish white. A prominent feature is the smell of butyric acid.

Excellent growth occurs in fluid media, with good turbidity and a heavy, white, clumpy or granular sediment, but no growth at the surface.

Biochemical reactions

Glucose: Acid and gas.
Maltose: Acid and gas.
Sucrose: Acid and gas.
Laevulose: Acid and gas.
Glycerol: Acid and gas.
Salicin: Acid and gas.
Indol: Negative.
Voges-Proskauer: Negative.
Nitrate reduction: Negative.
H_2S: Positive.
Gelatin liquefaction: Positive.
Litmus milk: Peptonised.
Coagulated sheep serum: Rapidly liquefied and blackened.
Coagulated egg albumin: Unattacked.
Cooked meat medium: Digested and blackened.
Catalase: Negative.
Peroxidase: Negative.
Haemolysis: Positive.
Fluorescence on MacConkey's medium: Positive.

The organism is non-pathogenic, and appears to be related to *Clostridium sporogenes*.

Type III. On agar this organism is a slender bacillus, 8μ or so long, with rounded ends and terminal or subterminal oval spores, arranged usually singly or in pairs. Spores are readily formed on all media. The organism is non-motile, and is Gram-positive in young cultures but soon becomes Gram-negative.

Colonies on agar are roundish, with a finely serrated edge and raised centre. The surface is finely pitted, with whitish, translucent colour and definite ground-glass appearance. There is no differentiation, and the colony is butyrous in consistency.

The organism grows well in all fluid media, giving good turbidity, a ropy sediment and a foul odour.

Biochemical reactions

Glucose: Acid and gas.
Maltose: Acid and gas.
Xylose: Acid and gas.
Dextrin: Acid and gas.
Sucrose: Acid and gas?
Laevulose: Acid and gas?
Indol: Negative.
Voges-Proskauer: Negative.
Nitrate reduction: Negative.
H_2S: Positive.
Gelatin liquefaction: Positive.
Litmus milk: Peptonised and digested to a clear fluid.
Catalase: Negative.
Peroxidase: Negative.
Haemolysis: Negative.
Fluorescence on MacConkey's medium: Positive.

Coagulated sheep serum: Rapidly liquefied to greenish fluid.
Coagulated egg albumin: No change.
Cooked meat medium: Much blackened and digested.

The relationship of this organism is unknown.

Several other organisms have occurred with various biochemical properties, e.g. fermentation of sugars, action on egg-medium, etc. They may be separate species—some, for example, are like the Uhrzeiger type of Pfeiffer, etc. (1917)—but pending more information on the serological relation of the non-pathogenic anaerobes, they have been included under one or other of the 3 types of group B. already described.

Of the types in group A, *C. putrificum* was found almost constantly in the intestines, but type III has occurred but once. It is of interest to note that, so far as is known, ours is the first recorded occurrence of *C. tetani* in the intestines of fish. In the course of this work this organism was first isolated at 20°C. It was at first thought to be an isolated occurrence, but later investigation showed that the organism is not infrequently present in haddock's intestines, and may be considered a member of the normal anaerobic flora there. The fact that the haddock feeds on the bottom, and that *Clostridium tetani* may be present in marine muds, etc., is sufficient to account for its occurrence, and is in accord with the ubiquitous nature of this particular organism.

The members of group B were always found in the haddock's intestines, and appear to constitute the major portion of the normal anaerobic flora there.

It is considered that the anaerobes play a rôle secondary to that of the aerobes in the decomposition (putrefaction) of fish, since the intestines are usually removed at sea soon after catching, and there are no anaerobes in the slime. There is little doubt, however, that without the removal of the intestines, and under certain other conditions, rapid decomposition of the proteins by the anaerobes would ensue. In his investigation of the decomposition of 75 fresh-water and marine fish, Schönberg (1930) also came to the conclusion that anaerobes play a minor part in the putrefaction of fish in commerical practice.

CONCLUSIONS

The occurrence of strict anaerobes in the slime and intestines of haddock from the North Sea was investigated.

No anaerobes were found in the slime, but various members of the group *Clostridium* were found in the intestines, including *Clostridium putrificum* or *Clostridium lentoputrescens* (Hartsell and Rettger), *Clostridium tetanoides* A (?), *Clostridium tetani* (recorded in the intestines of fish for the first time), *Clostridium bifermentans* (?), and *Clostridium sporogenes* (?).

The author desires to express his sincere thanks to Professor J. Cruickshank of the Bacteriology Department, University of Aberdeen for his constant help and advice during the course of this work, and also to Dr. MacLennan, of the same Department, for his work on the serological analysis.

REFERENCES

ADAMSON, R. S. 1918–19 On the cultural characters of certain anaerobic bacteria from war wounds. Jour. Path. and Bact., 22, 345–400.

CUNNINGHAM, A. 1932 The identity of *Bacillus putrificus* Bienstock. Jour. Bact., 24, 61–69.

FELLERS, C. R. 1926 Bacteriological investigation of raw salmon spoilage. Univ. Wash. Publ. in Fisheries, 1, 157–188.

HARTSELL, S. E., AND RETTGER, L. F. 1934 A taxonomic study of "*Cl. putrificum*" and its establishment as a definite entity—*Cl. lentoputrescens*, Nov. Spec. Jour. Bact., 27, 39–40, and 497–515.

HUNTER, A. C. 1920 Bacterial groups in decomposing salmon. Jour. Bact., 5, 543–552.

KIMURA, K. 1924 Zur Artbestimmung der Putrificus Bazillen. Centralbl. f. Bakt., Abt. I, Orig., 92, 342–356.

McCOY, E., AND McCLUNG, L. S. 1936 Studies on anaerobic bacteria. IX. Antigenic relations of *Clostridium bifermentans* and *Clostridium centrosporogenes*. Jour. Bact., 31, 557–568.

MORGAN, E. L., AND WRIGHT, H. D. 1934 *Clostridium putrificum* in association with post-operative tetanus. Jour. Path. and Bact., 39, 457–461.

OBST, M. 1919 A bacteriological study of sardines. Jour. Inf. Dis., 24, 158–169.

PFEIFFER, R., AND BESSAU, T. 1917 Ueber bakteriologische Befunde bei den Gasphlegmonen Kriegsverletzter. Deut. Med. Wochens., 43, (ii), 1217–1220, 1255–1258, and 1281–1285.

REDDISH, G. F. 1924 *Clostridium putrificum*. III. A comparison of strains obtained from collections in this country and abroad. Jour. Bact., 9, 321–326.

Reddish, G. F., and Rettger, L. F. 1922 *Clostridium putrificum* (*B. putrificus* Bienstock), a distinct species. Jour. Bact., 7, 505–510.

Reddish, G. F., and Rettger, L. F. 1923 *Clostridium putrificum*. II. Morphological, cultural and biochemical study. Jour. Bact., 8, 375–386.

Reed, G., and Spence, M. 1929 The intestinal and slime flora of the haddock—A preliminary report. Contrib. Canad. Biol. and Fisheries, New Series, 4, 257–264.

Schönberg, F. 1930 Über die Fischfäulnis und ihre bakteriologische Diagnose. Berlin Tierarzt. Wochensch., 46, 429–435.

Stewart, M. M. 1932 The bacterial flora of the slime and intestinal contents of the haddock (*Gadus aeglefinus*). Jour. Mar. Biol. Assoc., 18, No. 1, 35–50.

Topley, W., and Wilson, G. 1931 Principles of Bacteriology and Immunology. London.

Weinberg, M., and Ginsbourg, G. 1927 Données récentes sur les microbes anaerobies et leur rôle en pathologie. Monograph de l'Institut Pasteur.

Wilson, G. S. 1928 A modification of McIntosh and Fildes anaerobic jar. Jour. Path. and Bact., 31, 113–115.

EFFECTS OF SURFACE TENSION AND OSMOTIC PRESSURE ON THE GROSS MORPHOLOGY OF CERTAIN PATHOGENIC FUNGI[1]

JOHN W. WILLIAMS

Department of Biology and Public Health, Massachusetts Institute of Technology

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This work investigated surface tension and osmotic pressure as possible factors in variability of gross morphology of certain pathogenic fungi.

SURFACE TENSION

Surface tensions were taken on broths of mediums used with the du Noüy interfactial tensiometer at the air-broth interface; two minutes were allowed for stabilization after pouring.

Abbreviations are used: P, peptone Difco; D, glucose Difco; NT, sodium taurocholate Merck; GC, Gardinol Ca Dupont; BA, Brilliant Avirol L-124 Dupont; S, Sabouraud's medium of 1 per cent peptone, 4 per cent glucose; W, W medium of 4 per cent peptone, 1 per cent glucose; c, with.

The following medium slants (1.5 per cent agar Difco, pH 5.6) were studied (surface tension in dynes per cubic millimeter at 28C is given in parentheses): (a) 1 per cent P(62.8), (b) 4 per cent P(55.8), (c) 8 per cent P(52.7), (d) 12 per cent P(51.9) (e) 1 per cent P 1 per cent D(54.5), (f) W (52.3), (g) 8 per cent P, 1 per cent D (52), (h) 12 per cent P 1 per cent D (52), (i) S (56), (j) 4 per cent P 4 per cent D (53.9), (k) 8 per cent P 4 per cent D (53.2), (l) 12 per cent P 4 per cent D (52.9), (m) 1/4 per cent P 1/16 per cent D (63.4), (n) $\frac{1}{2}$ per cent P 1/8 per cent D (60.1), (o) 1 per cent P 1/4 per cent D (58.5), (p) 2 per cent P $\frac{1}{2}$ per cent

[1] Contribution No. 113 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

TABLE 1

Striking changes in gross morphology on lowering surface tension and increasing salt concentration in medium (increasing osmotic pressure)

	DECREASING SURFACE TENSION	INCREASING OSMOTIC PRESSURE
Achorion schoenleinii	Waxy, raised, spicules	Waxy, wet, irregular, raised, like Endodermophyton (79)
Acladium castellani	Petal-like, loss of color	Wet, waxy, nodular, loss of color, petal-like (101)
Candida candida	Not distinctive	Not distinctive (101)
Endodermophyton indicum	Waxy, wrinkled	Waxy, knob-like (31)
Endidermophyton tropicale	Waxy, knob-like	Waxy, subsurface mycelium (55)
Endomyces capsulatus	Coarser mycelia, waxy, raised centrally	Coarser mycelia, wetter (31)
Endomyces dermatitidus	Irregular, raised, wet, coarser mycelia	Waxy, lumpy, raised (55)
Epidermophyton inguinale	Raised centrally	Raised, lumpy, irregular (79)
Epidermophyton rubrum	Waxy, radiate, spicules heaped	Waxy, radiate, spicules, wrinkled, lumpy (55)
Glenospora gammeli	Waxy, raised	Knob of white mycelium (55)
Indiella americana	Wax paper-like, folded like Endodermophyton, irregularly radiate	Wax paper-like, raised centrally, irregularly radiate
Geotrichum bachmann	Not distinctive	Not distinctive (55)
Lichthemia sp.	Not distinctive	Wet and matted mycelium with 2.0 NaCl (101)
Microsporon audouini	Waxy, raised like teddy bear fur	Waxy, raised thicker mycelium (79)
Microsporon gypseum	Frosting, lumpy	Wrinkled (79)
Monosporium apiospermum	Irregular, waxy, heaped	Waxy (31)
Monilia albicans	Not distinctive	Not distinctive (101)
Oospora humi	Not distinctive	Not distinctive (55)
Scopulariopsis brevicaulis	Radiate, irregular, waxy	Radiate, irregular, waxy (101)
Sporotrichum schenckii	Irregularly scrambled	Irregularly scrambled, subsurface mycelium (55)
Trichophyton balcaneum	Increased growth, heaped	Thicker growth, irregular (55)
Trichophyton granulosum	Waxy, lumpy, spicules	Waxy, folded (79)

TABLE 1—*Concluded*

	DECREASING SURFACE TENSION	INCREASING OSMOTIC PRESSURE
Trichophyton gypseum-asteroides	Waxy, radiate	Waxy, raised, folded, irregular (104)
Trichophyton gypseum-lacticolor	Waxy, spicules, radii	Folded, wrinkled (79)
Trichophyton interdigitale	Raised, waxy, spicules, radii	Raised, irregular, radiate, resembles Trichophyton crateriforme and Epidermophyton inguinale (104)
Trichophyton japonicum	Not distinctive	Not distinctive (31)
Trichophyton niveum	Raised, frosted, spicules	Waxy, wrinkled (79)
Trichophyton purpureum	Waxy	Waxy (55)
Trichophyton sulfureum	Heaped, waxy, apricot skin appearance	Lumpy, waxy (79)
Willia anomala	Not distinctive	Not distinctive (104)

D (53.9), (q) 6 per cent P 1½ per cent D (51.6), (r) 8 per cent P 2 per cent D (50.1), (s) 12 per cent P 3 per cent D (50.1) (t) S c 0.5 per cent NT (39.9), (u) S c 0.25 per cent NT (42.7), (v) s C 0.125 per cent NT (43.7), (w) S c 0.05 per cent NT (50.1), (x) S c 0.005 per cent NT (53.4 per cent), (y) W c 0.5 per cent NT (40.3), (z) W c 0.25 per cent NT ((40), (aa) W c 0.125 per cent NT ((43.6), (bb) W c 0.05 per cent NT (49), (cc) W c 0.005 per cent NT (51.6), (dd) W c 0.5 per cent GC (43.6), (ee) W c 0.125 per cent GC (44.8), (ff) W c 0.005 per cent GC (47), (gg) W c 0.5 per cent BA (43.6), (hh) W c 0.125 per cent GC (44.8), (ii) W c 0.005 per cent BA (47.7), (jj) S c 0.5 per cent GC (43.6), (kk) S c 0.125 per cent GC (44.8), (ll) S c 0.005 per cent GC (48.8), (mm) S c 0.5 per cent BA (43.6 per cent), (nn) S c 0.125 per cent BA (45.1), (oo) S c 0.005 per cent BA (43.6).

Growths at room temperature in diffused light were observed for 45 days. Table 1 lists results produced or increased with lowering surface tension. These bore a significant relationship to the amount of lowering. Many of the morphological changes observed with variation of peptone to glucose are noted in previous papers (Williams 1935, a and b). Growths in concentrations greater than 4 per cent P, 1 per cent D (same ratio maintained)

were usually little changed and only slightly more profuse. With lower concentrations, changes were greater, growth on ¼ per cent P $\frac{1}{16}$ per cent D being proportionately more sparse and more subsurface. There is greater difference in dynes of surface tension in lower concentrations. In certain instances growth was somewhat decreased at lowest surface tension and with greater concentrations of GC, BA and NT. In the lower concentrations of BA, GC and NT growths were frequently increased over those on the base medium. Many organisms showed beautiful and characteristic changes. Controls without peptone showed irregular results which could not be correlated.

OSMOTIC PRESSURE

Osmotic pressure was raised by adding salts to W medium. The moles per liter of NaCl or KI added (resultant osmotic pressure of medium in atmospheres at 20°C. in parentheses) were: NaCl (a) 0.1 (11), (b) 0.3 (21), (c) 0.5 (31), (d) 1.0 (55), (e) 1.5 (79), (f) 2.0 (104), (g) 4.0 (199); *KI* (h) 0.1 (11), (i) 0.3 (21), (j) 0.5 (31).

Growth occurred on all slants of KI with frequently less growth at highest concentration. Table 1 lists atmospheric pressure in the highest salt concentration tube showing growth. As a rule, this and the previous tube or tubes showed decrease in growth. Osmotic pressure of W medium was 6.6 atmospheres at 20°C. as determined by fragility tests (that of Sabouraud's proof medium was approximately the same). This was added to osmotic pressures of salts. Agar was considered inert. Sodium chloride (3 moles) lowered surface tension to as much as 46.6 dynes which might play a part in results produced or increased presented in table 1. Several growths showed more abundant growth in the lowest concentration of salt than in the base medium. The highest osmotic pressure showed growth in none of the cases. Salt susceptibility effect is considered elsewhere (Williams, 1937b).

DISCUSSION AND CONCLUSIONS

Increasing concentration of peptone, adding sodium taurocholate, Brilliant Avirol L-124 or Gardinol Ca to W or S medium

decreased surface tension and produced or augmented the following appearances: colony delimitation, radii, frosting, wrinkling, raised appearance, waxiness, wetness, spicule, spike-like or whiskery mycelia. By lowering the surface tension of the S medium to that of the W medium duplication of W medium morphology was only partial. Findings indicate that surface tension may be an important factor in results.

Increasing osmotic pressure by adding sodium chloride or potassium iodide bore a proportionality relation and produced or augmented the following appearances: colony delimitation, raised appearance, irregularity of colony, waxiness, wetness and to a lesser degree other changes cited for decreased surface tension.

The least evaporation of moisture was from W medium containing 2 moles sodium chloride per litre; the amount was one-fourth less than for W medium. In view of other studies (Williams 1937c) this does not seem sufficiently great to explain results.

Certain organisms (*Candida candida, Geotrichum bachmann,* etc.) were stable to the effects of any of the agencies used.

Similarity of the progression of changes with decreasing surface tension and increasing osmotic pressure suggest a common underlying factor. This progression is evident: subsurface growth, surface growth, raised growth, first smooth then radiate, then irregular and lumpy growth. Previous studies have shown that on certain mediums (Williams, in publication) and with certain organisms sodium lactate and sodium phosphate can accomplish similar changes. Likewise, on certain mediums decrease in oxygen tension can produce some of these variations. In both of the latter instances surface tension and osmotic pressure do not seem to play a part.

In the use of the various agencies mentioned some organisms come to resemble each other. This may indicate a fundamental biological relationship.

Similarity of changes produced by increasing osmotic pressure and decreasing surface tension is responsible for inclusion of both problems in the same paper. Both seemingly accomplish a similar sequence of events. Whether the sequence has a physico-chemical basis can only be explained by further study.

REFERENCES

WILLIAMS, J. W. 1935a I. Effect of variation of ratios of dextrose to peptone on colonies of certain pathogenic fungi. Arch. Dermat. and Syph., 32, 893-914.

WILLIAMS, J. W. 1935b II. Effect of variation of ratios of dextrose to peptone on colonies of certain pathogenic fungi. Arch. Dermat. and Syph., 31, 15-34.

WILLIAMS, J. W. 1937a Allergy, immunity and growth of pathogenic fungi in vivo and in vitro. Urol. and Cutan. Rev., 41, 117-128; 198-201.

WILLIAMS, J. W. 1937b Effect of salts on certain pathogenic fungi. Arch. Dermat. and Syph., 36, 14-18.

WILLIAMS, J. W. 1937c Effect of relative humidity on gross morphology of certain pathogenic fungi. Proc. Soc. Exp. Biol. and Med., 37: 219-221.

WILLIAMS, J. W. Factors of importance in the production of surface growth of pathogenic fungi on culture mediums. Arch. Dermat. and Syph. To be published.

THE "TYROSINASE REACTION" OF THE ACTINOMYCETES

C. E. SKINNER

Department of Bacteriology and Immunology, University of Minnesota

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INTRODUCTION

The actinomycetes are classified sometimes with the bacteria and sometimes with the true fungi. They resemble the latter in that they possess a true branching mycelium and many species reproduce by the formation of conidia. They resemble bacteria in that they take bacterial stains (e. g. Gram, and, several species, also the Ziehl-Neelsen), no discrete nucleus has been proven, many species fragment and continue to reproduce by transverse fission, most species are not acid-tolerant as are the true fungi, and immunity reactions place them close to the tubercle bacillus (Henrici, 1930). Many species when grown on beef-peptone agar cause this medium to turn a dark brown to black color. This reaction was called the "tyrosinase reaction" by Lehmann and Sano (1908) because they found that the species studied by them produced this color only in media containing tyrosine. The pigment is thought to be melanin, known to be an oxidation product of tyrosine.

This dark color is very characteristic of the actinomycetes. Nearly one-third of the hundreds of strains isolated by the author in the past ten years, and not far from one-third of the species described by Waksman (1919) in his monographic treatment of the genus, and in later taxonomic treatises by others produced this coloration. It was noted by Gasparini (1891) who divided the genus *Actinomyces* into two aerobic species, *Actinomyces chromogenus* and *A. albus*. That *A. chromogenus* cannot be identified as one species is evident from the fact that a dark pigment discoloring the medium is recorded as being produced by no

fewer than seven species listed by Dodge (1935) and three species described by Erikson (1935), as animal pathogens. Most, if not all, of these are probably mere saprophytes, however. *Actinomyces scabies* (Thaxter) Güssow and other plant pathogens studied by Wollenweber (1920), by Millard and Burr (1926) and a host of saprophytes described and undescribed and differing from one another in morphology and physiology also produce this coloration.

Waksman (1916, 1919, 1920, 1930) threw doubt on the belief that the coloration was necessarily due to an oxidation of tyrosine. The reasons for this apparently well founded doubt may be summarized as follows: First, actinomycetes producing the dark color on beef peptone agar also produced it in media containing only gelatin and water. Gelatin was considered to contain no tyrosine. Secondly, a few species produced an apparently similar pigment on synthetic media containing no organic nitrogen; third, many "chromogenus" species, i.e., those which darken beef peptone agar, failed to produce a pigment on synthetic media to which tyrosine had been added. Pigments (green, red, violet, blue, yellow, etc.) are produced in various agar media by many species of actinomycetes, and slight changes in pH, or carbohydrate or nitrogen content of a medium will change the production of pigments, not only quantitatively but as to the color itself. So, implies Waksman very logically, it would not be surprising if others of the various pigments produced by actinomycetes, in most cases obviously totally unrelated to tyrosine metabolism, were dark brown or black.

Some of the true fungi are said to produce melanin from tyrosine-containing compounds (Dodge, 1919). Early work (Gessard 1898, 1901; Uyeda 1906; Carbone, 1907; Lehmann and Sano 1908; Lasseur and Thiery 1913) showed that the red color which develops and later turns brown in cultures of some strains of *Bacterium pyocyaneum* and other organisms, especially fluorescent bacteria, is due to tyrosine metabolism. Some strains of species of the genus *Rhizobium* were found to produce tyrosinase by Stapp (1923) and by Almon and Fred (1933). Not only was pigment produced only on tyrosine-containing media but when a

solution of tyrosine together with toluene was added to cultures a red color which turned black developed. Certain spirochetes, acidfast bacteria, and organisms causing brucellosis (Sanarelli, 1927; Battaglia, 1932; Mastroemi, 1932), have recently been found to produce a dark color from tyrosine.

Beijerinck (1900, 1911, 1913) studied the production of dark pigments by actinomycetes and other bacteria. He found that "*A. chromogenus*" produced the black pigment which he called melanin from peptone but not from free tyrosine, when one or the other of these substances was added to a medium. He thought the pigment was a catabolic product of the organic nitrogen. He also isolated several vibrios from sewage and found that an intense black color was produced from tyrosine. Actinomycetes were found also which did not produce color from free tyrosine when grown alone, but when these were grown with or close to certain bacteria which by themselves also were unable to produce pigments, an intense black color developed. Certain fluorescent bacteria also produced a "light red-brown pigment" from tyrosine. There have been very many other microörganisms described which produced a brown or black pigment in various media. A general study of the large number of species of "yeasts" such as *Monilia nigra* and various bacteria such as *Azotobacter chroococcum*, *Bacillus aterrimus*, *Bacillus niger*, etc., which are known to produce black or brown pigments, should be investigated as to the precursors of the pigments. It should be remembered that every black pigment is not necessarily "melanin," as anyone going over the large and scattered literature might be apt to conclude. One must assume that unless adequate chemical evidence is given, the identity of the pigment is unproven, irrespective of what name the particular author calls it.

EXPERIMENTAL

Eighty strains of actinomycetes giving dark brown to black pigment on beef peptone agar were isolated at random from plates of soil-extract agar poured from five samples of soil. A larger number which did not produce the pigment were also isolated. Hereafter in this paper, following the example of Krainsky (1914)

and Waksman (1916, 1919), the term "chromogenus" will apply to strains of actinomycetes which produce a definite brown or black pigment within a few days in beef peptone agar. All cultures were planted on a medium consisting of 15 per cent gelatin. All the eighty "chromogenus" strains which produced the pigment on the beef peptone agar also produced it in gelatin, and none of the "nonchromogenus" strains produced the color in gelatin. The same "chromogenus" strains also gave a brilliant reaction on a medium consisting of 15 per cent gelatin and 1.5 per cent agar, with no other ingredients. A much lighter color was shown by "chromogenus" strains on a medium consisting of only 1.5 per cent gelatin and 1.5 per cent agar. All the "nonchromogenus" strains in every case failed to darken the medium at all in the four days used for the test.

The gelatin used (Difco brand) gave a positive Millon test for tyrosine. In spite of the fact that many textbooks list gelatin as having no tyrosine, none of the many experienced biochemists consulted by the author, has ever found a sample of gelatin which failed to give a positive Millon test. In Morrow and Sandstrom's laboratory manual (1935), it is also implied that gelatin will give a faint Millon test. Bodansky (1930) lists gelatin as containing 0.01 per cent tyrosine. Stentström and Reinhard (1925) failed to find, by spectroscopic methods, any evidence of tyrosine in the sample of gelatin used by them. Unless they used a fairly concentrated solution of gelatin, the amount of tyrosine present—using Bodansky's figures taken from data which he considered most accurate—would probably not be detected by the method used. Possibly the common conception that gelatin is free of tyrosine is due to the fact that its presence is in such small amounts as to be negligible from a nutritional point of view. It is difficult to be certain just what pure gelatin really is unless it be crystallized, a feat which, according to some biochemists, has never been accomplished. At any rate, the results above, which exactly duplicate those of Waksman, do not indicate that the dark color is unrelated to tyrosine metabolism.

The cultures were inoculated on slants of Conn's (1921) agar (H_2O, agar-agar, K_2HPO_4, NaCl, sodium asparaginate, glycerol)

adjusted to pH 6.8 and 8.5, and on the same two media plus tyrosine to the concentration of 0.1, 0.01, 0.001, and 0.0001 per cent. In neither of the Conn's agar media without tyrosine was the black color formed, but some of the strains formed blue, violet, yellow, orange, or brown pigments in seven days in either or both the acid or basic Conn's agar media. In four days, usually in two or three days, all the "chromogenus" strains but none of the nonchromogenus strains formed a definite black color in all the 0.1 and 0.01 per cent tyrosine acid Conn's agar; and in the basic tyrosine medium the "chromogenus" strains either produced it, or, in the case of three strains, failed to grow at this alkaline reaction. In seven days, many of the strains of "chromogenus" actinomycetes showed a definite darkening in the medium containing 0.001 per cent tyrosine. Thus, there seems to be no doubt that the black color production in Conn's agar plus tyrosine is connected with tyrosine metabolism. Conn's medium, being white rather than yellow like beef peptone agar, shows the coloration as black rather than the dark brown noticed in beef peptone media.

But Waksman found that some of his "chromogenus" strains did not produce the dark pigment on a mineral-salt ammonium-sulphate glucose tyrosine agar. In my hands also this same medium failed to bring out the dark color with many of my "chromogenus" strains. Czapek's agar medium plus tyrosine likewise failed to develop the color when inoculated with many of the "chromogenus" actinomycetes. Czapek's medium, as used by Waksman (1919), contains various inorganic salts, sucrose and sodium nitrate. Also, the color was produced by none of my "chromogenus" strains on Conn's agar plus tyrosine minus both glycerol and sodium asparaginate, and by only a few of the "chromogenus" strains on Conn's agar minus sodium asparaginate but with glycerol and tyrosine. Thus, it seems that any medium supporting growth to which tyrosine has been added is not suitable for formation of the pigment. Conn's complete medium plus tyrosine, however, in every case produced the pigment, but only with "chromogenus" actinomycetes, and with all strains of these.

Waksman's second objection, namely that a brown color is produced in certain non-tyrosine-containing media, by some strains of "chromogenus" or "nonchromogenus" actinomycetes was also investigated. Some of the "chromogenus" strains, when grown on unmodified Czapek's agar particularly, but to a less extent in Conn's unmodified agar, showed in seven days a brown pigment which deepened on further incubation. The pigments were not formed in four days on these nontyrosine media, or in a few cases there was only a slight yellow tint, at which time the tyrosine-containing media showed a much deeper and darker color. The same results as to pigment production on non-tyrosine-containing media were found by Conn and by Waksman with *Actinomyces pheochromogenus* Conn and *Actinomyces purpeochromogenus* Waks., and by Waksman with *Actinomyces diastaticus* Waks. The latter organism produced no pigment on beef peptone agar. That this brown pigment produced on long incubation is not the same as the darker pigment produced from tyrosine-containing media by "chromogenus" strains in a few days, was indicated in the following way.

Several of these "nonchromogenus" strains, i.e., those which failed to darken beef peptone agar, but which produced the brown pigment on non-tyrosine-containing synthetic media, were plated on tyrosine-free agar medium (Czapek's and Conn's) and "chromogenus" strains were plated on Conn's tyrosine agar in flasks and all incubated for from ten days to two months. Alcohol, water, or chloroform were added to flasks of each. It was found that the brown pigment in Czapek's and Conn's agar was quickly soluble in water and alcohol, whereas the black pigment in the tyrosine Conn medium took hours to go into solution and then only slightly. The brown color in the non-tyrosine media was slightly soluble in chloroform and the black pigment in Conn's tyrosine medium, not at all.

A different coloration by a few "nonchromogenus" strains, only in tyrosine-containing media was noted. Some of the strains which had no effect as far as color change was concerned in beef peptone or in non-tyrosine-containing synthetic media turned the tyrosine media (Conn's or Czapek's) a distinct pink

to red color in five to ten days. This often darkened slowly to a dark brown. This darkening was most rapid in the basic media. This is the color change of the enzyme "tyrosinase" (Bodansky, 1930) of the chemists. It may be that the "tyrosinase reaction" known to the bacteriologists in relation to the actinomycetes really consists of three enzymes: (1) hydrolysing the peptone to tyrosine, (2) oxidizing the tyrosine to Raper's red compound, and (3) oxidizing this to melanin. The organisms producing this red color from free tyrosine, then, must lack the first enzyme, and either lack the third or produce a very small amount of it. The second reaction has been shown by Raper (Bodansky, 1930) to consist of four distinct steps, and there may be more than one enzyme involved in this step alone. It is often thought that Raper's red compound changes slowly to melanin, spontaneously, especially under basic conditions, but Gortner (1924) found that an enzyme was necessary to oxidize the red compound to melanin. And it may be that there are different "tyrosinases" among the actinomycetes, as thought by some to occur elsewhere in nature. But all this in relation to microorganisms is at present mere speculation.

Apparently other amino acids than tyrosine do not ordinarily give rise to this black color. Conn's agar medium to which 0.1 per cent alanine, cystine, glycine, histidine dihydrochloride, phenylalanine, or tryptophane, were added, failed to develop the black color noted when inoculated with either the "chromogenus" or "nonchromogenus" actinomycetes. All of the "chromogenus," but none of the "nonchromogenus" strains caused a darkening of Conn's medium in four days or less if 1 per cent casein or edestin was incorporated in the medium. These proteins, of course, contain tyrosine. Several of the "nonchromogenus" strains when inoculated on Conn's agar to which phenylalanine was added developed a red or pink color which turned brown to black on standing. The strains causing this change were mostly the same strains noted above, which failed to darken beef peptone agar but which turned Conn's tyrosine agar first pink, then a brown to black color. Likewise, a few strains turned only media containing histidine, and one strain only tryptophane-

containing media, a bright yellow. It may be that many of the color changes of the actinomycetes that have been so much studied by Krainsky (1914), Waksman and others are results of *action on special amino acids.*

Waksman's observations on the production of dark brown or black pigments among the actinomycetes have been confirmed *in all details,* but his conclusions need to be modified by the further results here reported. It seems evident from the data that the term "tyrosinase reaction" is a correct one, although many enzymes are possibly involved, and that the color change on certain media so characteristic of so many of the actinomycetes is truly due to tyrosine metabolism.

Work is now in progress on the study of the black and brown pigments of actinomycetes and other microorganisms by means of chemical and physical methods in collaboration with Dr. E. Arnow of the Department of Physiology and Physiologic Chemistry. Also the identity of the red pigment produced by many "nonchromogenus" actinomycetes and other bacteria in tyrosine and phenylalanine media is being investigated.[1]

CONCLUSION

Inasmuch as the same strains of actinomycetes which produced a dark brown to black pigment on beef peptone or protein-containing media were the only strains which produced the characteristic coloration in a few days, in a synthetic medium to which tyrosine was added, and failed to produce it unless this particular amino acid was added, and since the seemingly anomalous results of others have been explained, it is concluded that the rapid production of a dark color by "chromogenus" actinomycetes is due to tyrosine metabolism."[2]

REFERENCES

ALMON, LOIS, AND FRED, E. B. 1933 The production of tyrosinase among various species of Rhizobium and related organisms. Zent. Bakt. II, 88, 302–304.

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[1] The work here reported as well as that now in progress has been aided by a grant from the Research Funds of the Graduate School.

[2] A preliminary report of this paper was published as a detailed summary, Jour. Bact., 34, 136–137, 1937.

Battaglia, M. 1932 Origine du pigment noir dans les cultures l' "Eumices" tuberculosis. Bol. Sez. Ital. Soc. Int. Microb., 4, 410–412.

Beijerinck, M. W. 1900 Sur la production de quinone par le Streptothrix chromogena et la biologie de ce microbe. Arch. Neérl. Sci. Ex. Nat. Ser. 2, 3, 327–340.

Beijerinck, M. W. 1911 Pigments as products of oxidation by bacterial action. Proc. Sec. Sci. Kon. Akad. Wetenschappen, Amsterdam, 13, 1066–1077.

Beijerinck, M. W. 1913 On the composition of tyrosinase from two enzymes. Proc. Sec. Sci. Kon. Akad. Wetenschappen, Amsterdam, 15, 932–937.

Bodansky, Meyer. 1930 Introduction to Physiological Chemistry. 2nd edition. Wiley, New York.

Carbone, D. 1907 Ricerche su l'origine di alcuni pigmenti microbici con speciale riguardo a la tirosinasi. Zent. Bakt. II, 19, 587.

Conn, H. J. 1921 The use of various culture media in characterizing Actinomyces. N. Y. Agr. Exp. Sta. (Geneva) Tech. Bull. 83.

Dodge, Carroll W. 1919 Tyrosin in the fungi: chemistry and methods of studying the tyrosinase reaction. Ann. Mo. Bot. Gard., 6, 71–92.

Dodge, C. W. 1935 Medical Mycology. Mosby, St. Louis.

Erikson, Dagny. 1935 The pathogenic aerobic organisms of the Actinomyces group. Med. Res. Council (British). Sp. Report Ser. 203.

Gasparini, G. 1891 Ricerche morphologiche e biologiche sul genera Actinomyces Harz. Ann. Inst. Ig. Sper. Univ. Roma, 2, 167–299 (original not seen).

Gessard, C. 1898 Sur une propriété nouvelle du bacille pyocyanique. Compt. Rend. Soc. Biol., 50, 1033–1035.

Gessard, C. 1901 Variété melanogène du bacille pyocyanique. Ann. Inst. Past., 15, 817–833.

Gortner, Ross Aiken. 1924 Observations on the mechanism of the tyrosine-tyrosinase reaction. Proc. Soc. Exp. Med. Biol., 21, 543–544.

Henrici, Arthur T. 1930 Molds, Yeasts and Actinomycetes. Wiley, New York.

Krainsky, A. 1914 Die Aktinomyceten und ihre Bedeutung in der Natur. Zent. Bakt. II, 41, 649–688.

Lasseur, P. H., and Thiery, G. 1913 Sur les cultures colorées de bacteries considérées jusqu' a present comme achromogènes. Compt. Rend. Acad. Sci., 156, 166–168.

Lehmann, K. B., and Sano 1908 Über das Vorkommen von Oxydationsfermenten bei Bakterien und höheren Pflanzen. Arch. Hyg., 67, 99–113.

Mastroeni, Marco 1932 La chromogènèse chez les germes du type "Brucella" dans un milieu à l'oeuf. Bol. Sez. Ital. Soc. Int. Microb., 4, 268–272.

Millard, W. A., and Burr, Sydney 1926 A study of twenty-four strains of Actinomyces and their relation to types of common scab of potato. Ann. Appl. Biol., 13, 580–644.

Morrow, C. A., and Sandstrom, W. M. 1935 Biochemical Laboratory Methods. Wiley, New York.

Sanarelli, G. 1922 Les spirochètes caecaux. Ann. Inst. Past., 41, 1–44.

Stapp, C. 1923 Beiträge zum Studium des Bakterientyrosinase. Biochem. Ztschr., 141, 42–69.

Stenström, Wilhelm, and Reinhard, Melvin 1925 Ultraviolet absorption spectra of blood serum and certain amino acids. Jour. Biol. Chem., 66, 819-827.

Waksman, Selman A. 1919 Cultural studies of species of Actinomyces. Soil Sci., 8, 71-207.

Waksman, Selman A. 1920 Studies in the metabolism of actinomycetes. III. Nitrogen metabolism. Jour. Bact., 5, 1-30.

Waksman, Selman A. 1930 Principles of Soil Microbiology. 2nd edition. The Williams & Wilkins Co., Baltimore.

Waksman, Selman A., and Curtis, Roland E. 1916 The actinomyces of the soil. Soil Sci., 1, 99-134.

Wollenweber, H. W. 1920 Der Kartoffelschorf. Arb. Forschungsinst. Kartoffelbau, Berlin, 2, 1-102.

Uyeda, Y. 1906 Bacillus Nicotianae, sp. nov., die Ursache der Tabakwelkkrankheit oder Schwarzbeeinigkeit in Japan. Bul. Imp. Centr. Agr. Exp. Sta. Japan (4-21), 1, 39-55. (Original not seen.)

THE SEROLOGICAL IDENTIFICATION OF STREPTOCOCCUS ZYMOGENES WITH THE LANCEFIELD GROUP D

F. R. SMITH, C. F. NIVEN AND J. M. SHERMAN

Laboratories of Bacteriology and Dairy Industry, Cornell University, Ithaca, New York

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In their study of *Streptococcus zymogenes*, Sherman, Stark and Mauer (1937) stated that on the basis of a physiological study of a few of Dr. Lancefield's cultures, the Lancefield group D hemolytic streptococcus and *Streptococcus zymogenes* appeared to be the same. There was nothing essentially new in this suggestion: Lancefield and Hare (1935) had previously stated that the group D streptococci were in fact more closely related to *Streptococcus fecalis* than to *Streptococcus pyogenes*; Hare and Maxted (1935) isolated group D streptococci from human feces and related them to the "hemolytic enterococci" of previous workers; and Houston (1936) had mentioned *Streptococcus zymogenes* as one of the enterococci which belong in one serological group by the Lancefield method.

As the studies in this laboratory of the members of the enterococcus division of the streptococci have been somewhat more detailed with reference to the identity of the several "species" belonging to this group, than have most of the papers which have dealt somewhat loosely with "enterococci," it seemed desirable to classify by the Lancefield precipitin technique a collection of these more extensively studied cultures which have been specifically identified as *Streptococcus zymogenes*. For this purpose, 76 cultures have been studied, 70 of these being isolated from human feces and six from pasteurized milk.

SEROLOGICAL FINDINGS

For the production of antisera, in the preparation of bacterial extracts for the precipitin tests, and in performing the tests,

the methods described by Lancefield (1933) were faithfully followed.

In our hands, good group D antiserum has been more difficult to produce than have sera for most of the other standard Lancefield groups. We have, however, obtained some excellent group D antisera after five courses of treatments, each consisting of a seven-day course of daily injections followed by a seven-day rest period. The results reported in this paper were obtained with two different antisera, both of which, however, were produced by using the same culture (26Cl) as the immunizing agent. This culture was one of our own typical strains of *Streptococcus zymogenes* (hemolytic and proteolytic) which Dr. Lancefield had very kindly identified as belonging to group D.

The results may be briefly stated: All of the extracts made from the 76 cultures which had been identified as *Streptococcus zymogenes*, by physiological studies, gave good reactions with both of the antisera used. As a further control on the integrity of our own results it may be mentioned that two of Dr. Lancefield's group D strains were included in the tests, and the extracts from each of them gave strong reactions with both of the antisera used.

PHYSIOLOGICAL CHARACTERISTICS OF CULTURES

In order that the nature of the organisms studied may be clear, their physiological characteristics are given.

All of the cultures used in this study were hemolytic in blood agar, all grew at 10°C., and all except one grew at 45°C. All grew in the presence of 6.5 per cent sodium chloride, in alkaline media with pH values of 9.6, and in 0.1 per cent methylene blue in skimmed milk. All survived heating at 60°C. for thirty minutes in skimmed milk. All cultures produced a final pH of 4.4 to 4.0 in glucose broth, produced ammonia in 4 per cent peptone, and split esculin. Starch was not hydrolyzed by any of the cultures, and only one of the cultures hydrolyzed sodium hippurate.

Forty of the cultures had strong reducing action as shown by the reduction of litmus milk before curdling, while 36 strains

reduced litmus after the milk was curdled. Although strong reducing action (revealed by the before-curdling reduction of litmus in milk cultures, and by other tests) is usually one of the most characteristic properties of *Streptococcus zymogenes* and its near relative, *Streptococcus fecalis*, strains belonging to this general group are sometimes atypical in this respect (Long and Hammer, 1936; Sherman, Mauer and Stark, 1937). The ability to liquefy gelatin has usually been considered a general characteristic of *Streptococcus zymogenes*, but Sherman, Stark and Mauer (1937) concluded that non-proteolytic strains of this organism should be recognized. In the present study 16 of the cultures liquefied gelatin whereas 60 appeared to be entirely non-proteolytic as revealed by gelatin and milk cultures.

In addition to glucose, all of the cultures fermented maltose, lactose, trehalose, mannitol, sorbitol, and salicin. Fermenting and non-fermenting strains were found with sucrose (52+, 24−) and glycerol (72+, 4−). None of the cultures fermented arabinose, xylose, raffinose, nor inulin.

DISCUSSION

The present work correlates previous physiological and serological findings and identifies more precisely at least one member of the Lancefield group D. All "hemolytic enterococci," in the loose sense, are not *Streptococcus zymogenes*, but recent studies in this laboratory indicate that it is the most common form in the human intestine; the only other fecal hemolytic streptococcus, thus far encountered, which might properly be considered an enterococcus, is the closely related, but physiologically distinct, *Streptococcus durans* (Sherman and Wing, 1937). It is important to know whether or not the Lancefield group D includes other closely related hemolytic types, such as *Streptococcus durans*, in addition to *Streptococcus zymogenes*. Work is now under way on this problem.

With reference to the disputed point as to whether or not non-proteolytic strains of *Streptococcus zymogenes* should be recognized, the data here reported have some pertinence. The serological results support previous physiological findings in

indicating that the proteolytic and non-proteolytic strains belonging to this general type are otherwise essentially the same, if not identical. It is of interest to know, contrary to previous indications, that non-proteolytic strains appear to be the prevailing type.

SUMMARY

Seventy-six cultures of *Streptococcus zymogenes* which had been identified by physiological methods were studied by means of the Lancefield serological technique. All of these cultures were found to belong to the Lancefield group D.

REFERENCES

Hare, Ronald, and Maxted, W. R. 1935 The classification of haemolytic streptococci from the stools of normal pregnant women and of cases of scarlet fever by means of precipitin and biochemical tests. Jour. Path. Bact., 41, 513–520.

Houston, T. 1936 Enterococci. Second International Congress for Microbiology. London. Report of Proceedings, 141–142.

Lancefield, Rebecca C. 1933 A serological differentiation of human and other groups of hemolytic streptococci. Jour. Exp. Med., 57, 571–595.

Lancefield, Rebecca C., and Hare, Ronald 1935 The serological differentiation of pathogenic and non-pathogenic strains of hemolytic streptococci from parturient women. Jour. Exp. Med., 61, 335–349.

Long, H. F., and Hammer, B. W. 1936 Classification of the organisms important in dairy products. I. *Streptococcus liquefaciens*. Iowa Agr. Expt. Sta., Res. Bul. 206.

Sherman, J. M., Mauer, J. C., and Stark, Pauline 1937 *Streptococcus fecalis*. Jour. Bact., 33, 275–282.

Sherman, J. M., Stark, Pauline, and Mauer, J. C. 1937 *Streptococcus zymogenes*. Jour. Bact., 33, 483–494.

Sherman, J. M., and Wing, Helen U. 1937 *Streptococcus durans* N. Sp. Jour. Dairy Sci., 20, 165–167.

EXPERIMENTS ON THE NUTRITION OF STREPTOCOCCI

S. H. HUTNER

Laboratory of Bacteriology, College of Agriculture, Cornell University, Ithaca, New York

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The identification of the indispensable nutritional requirements of the streptococci has been the object of few investigations, despite the importance of the group and a number of clear-cut early advances. Hosoya and Kuroya (1923) discovered that a pyogenic streptococcus needed something accompanying vitamin B_1 in an alcoholic extract of rice bran. This factor was stable to heat and acid; destroyed by drastic alkali treatment; adsorbed by fuller's earth; and precipitated by phosphotungstic acid. Freedman and Funk (1922), using the same hemolytic strain studied by Mueller, obtained similar data. Beef-heart infusion decolorized by norite charcoal allowed no growth, but was reactivated by the addition of peptone. Peptone alone did not permit growth. A streptococcus factor removed from autolyzed yeast by fuller's earth could be eluted with $Ba(OH)_2$. Mueller (1922) found that norite-inactivated meat infusion was reactivated by the addition of acid-hydrolyzed casein as well as by peptone. One of the essential amino acids in the hydrolysate was identified as methionine. Another, found in the "monoamino" fraction of the Dakin butyl alcohol separation, and precipitated by $HgCl_2$ in 5 per cent H_2SO_4 and by Ag_2SO_4, was not identified. Some additional properties of the hemolytic streptococcus factor were recorded: presence in spinach and blood; unextractibility by ether; stability toward oxidants and reductants.

Orla-Jensen and associates (1936a, b) recently reported their extensive study of the nutrition of saprophytic streptococci. They found that the active material in skim milk, adsorbed onto

fuller's earth or norite, was eluted by a pyridine-methanol mixture. Deproteinized milk was made a better medium by the addition of casein. Riboflavin was viewed as essential. Several amino acids were found favorable, although ammonium salts sufficed as sole nitrogen source. Evaluation of these data is rendered difficult by the absence of details of the methods used for minimizing contamination of experimental media with material carried over from stock cultures. The present brief investigation developed from attempts to extend the work from Orla-Jensen's laboratory, and was as much aimed at finding the best species for experimental work as at identifying some of the growth essentials.

CULTURAL TECHNIQUE

The basic medium consisted of inorganic salts (NH_4Cl 0.05 per cent; $MgSO_4 \cdot 7H_2O$ 0.05 per cent; K_2HPO_4 0.033 per cent; KH_2PO_4 0.017 per cent; $FeSO_4 \cdot 7H_2O$ approximately 0.001 per cent) plus Na citrate·11/2 H_2O 0.5 per cent and sucrose 2.0 per cent. The citrate served as buffer and to minimize heavy metal toxicity. Sucrose was chosen because it can be autoclaved without decomposition. Only sucrose-fermenting streptococci were used. The initial pH of all media was adjusted to 7.0 to 7.3. Cultures were maintained in 125 cc. Pyrex Erlenmeyer flasks containing 35 cc. of medium. Concentrations of nutrients were calculated, however, on a basis of a 25 cc. volume of medium so that all culture fluids were more dilute by the factor 5/7. Sterilization was by autoclaving for 10 minutes at 115°C. No evidence was found of destruction of growth essentials by this treatment. Growth was measured by the number of cubic centimeters of 0.1 N NaOH necessary to titrate a culture to pinkness with phenolphthalein after subtraction of the alkali expended on an uninoculated duplicate flask. Cultures were incubated at least 24 hours at 37°C. The stock broth used in most of this work had the following composition: Difco beef extract 0.3 per cent; Difco peptonized milk 0.5 per cent; Difco yeast extract 0.5 per cent; Difco tryptone 0.1 per cent; glucose 0.2 per cent; and $CaCO_3$ in excess. The slight, or no, growth in

control flasks made up of the basal solution plus adequate amino acids in the form of acid-hydrolyzed casein indicated that gross carry-over effects from the one-drop inocula were negligible. Eastman organic chemicals were used.

CHOICE OF ORGANISMS

Streptococcus liquefaciens was selected as our initial experimental object because of its good growth in peptone and exceptional resistance for a streptococcus to extreme physical conditions. Unfortunately the strain used, No. 805, produced so much alkali in deproteinized milk or yeast media that titration figures did not match growth. A strain of the closely related *Streptococcus zymogenes* (No. In1) was found free of this objection but was studied only at the close of the investigation. The following vigorously-growing forms were selected:

Streptococcus bovis No. 11. Isolated by Mrs. C. N. Stark from the mouth of cattle.

Streptococcus inulinaceous. Ditto.

Streptococcus asalignus (*nina*). Originally isolated by Prof. Frost.

Streptococcus mastitidis No. 1P1. Isolated by Dr. F. R. Smith from normal human feces and giving the precipitin test for Lancefield Group B.

The Dochez "N. Y. 5" strain of "*pyogenes*" was chosen in preference to the typical "*pyogenes*" described by Evans (1936) because of its greater hardiness and more abundant growth both in skim milk and yeast media.

Titration values for the forms enumerated were a satisfactory index of growth.

AMINO ACID REQUIREMENTS

The ease and thoroughness with which the protein can be removed from skim milk, leaving very little protein behind, made skim milk thus treated efficient in detecting amino acid requirements. The good growth most streptococci make in skim milk imply it to be a good source of their vitamins. Furthermore it is cheap and available, and its non-protein bulk consists largely of

readily separable ash and lactose. Following Orla-Jensen's lead, skim milk fractions were made the basis of experimental work. Okuda and Zeller's (1921) directions for maximal protein removal were followed: a suspension of skim milk powder in water at pH 4.5 was steamed 10 minutes at 100°C. The protein coagulum was filtered off and washed with acidulated hot water. The combined filtrate and washings were brought to neutrality with NaOH and the calcium phosphate precipitate removed. The resulting deproteinized skim milk (PFM) was preserved with chloroform, as were all other putrescible solutions. No loss in activity was detected in the preparation after several months at room temperature in darkness.

TABLE 1

Growth of S. pyogenes in deproteinized milk and hydrolyzed casein

	NaOH
	cc.
PFM 1.0 per cent	0.9
PFM 1.0 per cent plus AHC 0.5 per cent	3.3
PFM 2.0 per cent	0.4
PFM 2.0 per cent plus AHC 0.5 per cent	4.4

A sulfuric acid hydrolysate of casein (AHC) was prepared in the usual manner from Pfanstiehl's "vitamin-free" product.

S. pyogenes makes a relatively poor growth in PFM plus AHC as compared with its growth in unaltered skim milk. A likely explanation is that in the process of protein removal serious amounts of growth factors are lost by adsorption on the protein. Freedman and Funk (1922) in fact noted that hydrolysates of ordinary unpurified proteins allowed growth while hydrolysates of purified proteins did not. Tests with PFM clearly showed dependence upon amino acids (table 1).

The poor and somewhat irregular growth of this streptococcus in these media led to a comparison between PFM and yeast extract (Difco) as vitamin sources, all cultures being supplied with AHC at the 0.5 per cent level. The results are given in table 2.

There was clear evidence of complementary action—an indication of multiple factors. Other experiments proved that tryptophane was not a factor in the stimulation by yeast. Because of this complexity of growth factors, other streptococci growing far more vigorously in deproteinized milk were studied. It was always easy to elicit the casein stimulation (table 3).

TABLE 2

Growth of S. pyogenes in deproteinized milk and yeast extract

	NaOH
	cc.
AHC alone	0.0
PFM 0.5 per cent	1.1
PFM 1.0 per cent	1.0
Yeast 0.1 per cent	0.5
Yeast 0.5 per cent	0.2
PFM 0.5 per cent plus yeast 0.1 per cent	3.0

TABLE 3

Growth of streptococci in deproteinized milk, yeast, and hydrolyzed casein

	NaOH			
	S. asa.	*S. bov.*	*S. inu.*	*S. mas.*
	cc.	*cc.*	*cc.*	*cc.*
PFM 2.0 per cent	0.3	0.5	1.3	0.3
PFM 2.0 per cent plus AHC 0.5 per cent	8.4	6.3	7.7	8.4
PFM 2.0 per cent plus yeast 0.25 per cent plus AHC 0.25 per cent	8.8	9.1	12.3	8.8
Yeast 0.25 per cent plus AHC 0.25 per cent	1.4	10.3	7.0	1.7

It will be noted that *S. mastitidis* and *S. asalignus* grow poorly in yeast. This is in marked contrast to *S. bovis* and the closely related *S. inulinaceous*, the first of which grows as luxuriantly as *S. liquefaciens* in milk or yeast media.

A similar effect of hydrolyzed casein was found for *Streptococcus liquefaciens*, *Streptococcus zymogenes*, *Streptococcus durans* and *Streptococcus salivarius*.

Attention was then directed to the identification of the essen-

tial amino acids in the hydrolyzed casein. Cystine (furnished in slight excess) was found partly to replace hydrolyzed casein (table 4). Cystein hydrochloride (0.01 per cent), sterilized and added separately to avoid decomposition, was as effective. Trial of cystine was suggested by its sharing the property of Mueller's unidentified amino acid of being precipitated by acid mercuric reagents. The nature of the amino acid deficiencies coming into play after the satisfaction of the cystine requirement was the subject of a few experiments. Supplements of methionine, tyrosine, leucine and phenylalanine together were entirely ineffective for the three streptococci tested: *S. asalignus*, *S. bovis* and *S. mastitidis*. Occasionally growth with cystine would almost

TABLE 4

Effect of replacement of casein hydrolysate by cystine

	NaOH		
	S. asa.	*S. bov.*	*S. mas.*
	cc.	*cc.*	*cc.*
PFM 1.0 per cent	0.0	0.2	0.0
PFM 1.0 per cent plus cystine	0.4	2.3	1.5
PFM 1.0 per cent plus AHC 0.25 per cent	4.4	4.4	7.2

equal that with the casein hydrolysate but on serial subculture growth fell off almost entirely in the cystine flasks, but hardly at all in those with the hydrolysate.

STREPTOCOCCUS VITAMINS

Mueller (1922) reported disappointing results with heavy metal precipitation procedures for concentrating the *S. pyogenes* factor. It was felt that the complexity of the requirements of *S. pyogenes* might have been partly responsible, and that work with less exacting streptococci might yield clearer data. *S. asalignus*, *S. bovis* and *S. mastitidis* were used.

Heavy metal precipitations. One liter of PFM was evaporated on a steam bath under an electric fan to 250 cc., the temperature not rising above 75°C. This concentrate was used for each

precipitation. An untreated portion of concentrate served as control.

(a) *$Ba(OH)_2$—ethanol.* Fifty cubic centimeters of concentrate were chilled to 10°C. Hot saturated $Ba(OH)_2$ was added in equal volume with vigorous stirring and, immediately after, an excess (400 cc.) of chilled 95 per cent ethanol. The curdy precipitate was placed in the refrigerator and shaken from time to time to prevent caking. After six hours the precipitate was filtered off with the aid of "filter-cel" (also used in the other precipitations) and washed with 95 per cent ethanol. Alcohol was removed from the filtrate by evaporation on a steam bath under electric fan. Barium was removed with H_2SO_4. On evaporation of an aliquot of the regenerated original barium precipitate abundant crystals of lactose formed.

(b) *Lead acetate—$Ba(OH)_2$.* Fifty cubic centimeters of concentrate were treated with saturated basic lead acetate until no further precipitation occurred, then brought to pH 8.6 by the addition of cold saturated $Ba(OH)_2$. After standing overnight the precipitate was filtered off and washed with water. It was then decomposed with a slight excess of H_2SO_4, the $PbSO_4$ and $BaSO_4$ filtered off, and the lead remaining in the filtrate removed with H_2S. The PbS precipitate was washed with boiling water. H_2S was removed from the PbS filtrate by bringing the solution to a boil. The original Pb-Ba filtrate was similarly treated.

(c) *$HgSO_4$.* Fifty cubic centimeters of chilled concentrate was treated with 25 cc. of West and Peterson's mercury reagent (a saturated solution of $HgSO_4$ in 10 per cent H_2SO_4). Warm saturated $Ba(OH)_2$ was added in small portions after successive chillings so that room temperature was not exceeded. When pH 7.0 was reached the flask was set overnight in the refrigerator, the combined mercury and $BaSO_4$ precipitate filtered off and washed with small portions of ice water. The precipitate and filtrate were worked up as in the lead precipitation.

(d) *Copper-lime.* Fifty cubic centimeters of concentrate were treated with 25 cc. of 25 per cent $CuSO_4$. An excess of $Ca(OH)_2$ suspension was added and the resulting bulky precipitate broken

up into a fine suspension. The precipitate was filtered off and washed thoroughly with saturated $Ca(OH)_2$ and was then decomposed with a slight excess of oxalic acid. H_2S was passed in. The combined calcium oxalate and CuS precipitates were filtered off and washed with boiling water. The oxalate remaining in the filtrate was removed with $CaCO_3$. The original filtrate was worked up in the same way as the precipitate.

TABLE 5

Growth of streptococci in heavy metal fractions of deproteinized milk plus 0.5 per cent casein hydrolysate

	NaOH		
	S. asa.	*S. bov.*	*S. mas.*
	cc.	*cc.*	*cc.*
AHC alone	1.4	0.3	1.4
PFM 0.5 per cent	6.9	9.4	5.3
Ba filtrate	1.7	7.2	2.6
Ba precipitate	2.8	0.3	4.9
Ba 0.5 per cent each together	5.4	6.7	5.7
Pb filtrates	0.3	0.3	3.0
Pb precipitate	2.5	5.1	3.6
Pb 0.5 per cent together	1.0	8.3	5.7
Hg filtrate	0.1	2.6	1.9
Hg precipitate	1.0	0.2	3.5
Hg 0.5 per cent each together	2.9	8.0	4.1
Cu filtrate	0.3	3.4	1.2
Cu precipitate	6.3	5.0	2.2
Cu 0.5 per cent each together	5.3	6.1	4.6

The growth tests were conducted with a concentration of each fraction corresponding to 0.5 per cent of the original deproteinized milk, considering only the organic matter content, as in all other experiments. This concentration of PFM allows about two-thirds maximal growth. The results are shown in table 5.

The data are clearer for *S. bovis* than for *S. asalignus* and *S. mastitidis*. The mercury precipitation data point to multiple factors, one of them a base. Later adsorption experiments sup-

port the belief that the principal loss of growth factors was by adsorption on the sulfide precipitates rather than by actual destruction. Fractionation with silver nitrate-$Ba(OH)_2$ was attempted but the very low activity of the fractions for *S. bovis* and *S. mastitidis*, the only strains tested, discouraged additional work.

Fuller's earth adsorption Adsorption experiments with fuller's earth gave rise to the belief that all streptococci require at least one basic factor which may be characteristic for the genus. A large number of experiments were carried out with Eimer and Amend's fuller's earth and "Frankonite KL." Several other brands, in contrast, were quite inactive. The following is a

TABLE 6

Growth in fuller's-earth-treated deproteinized milk

	NaOH		
	S. asa.	*S. bov.*	*S. mas.*
	cc.	*cc.*	*cc.*
PFM untreated	7.1	8.5	5.4
Fuller's earth 0.4 per cent	7.8	6.3	6.7
Fuller's earth 2.0 per cent	1.8	1.5	2.9
Fuller's earth 10.0 per cent	0.3	1.7	2.7

sample experiment: deproteinized milk was brought to pH 4.3 and treated with the following concentrations of Frankonite KL: 0.4, 2.0 and 10 per cent. The filtrates were brought back to neutrality and tested in concentrations corresponding, as in previous experiments, to 0.5 per cent of the original deproteinized milk. All cultures were with 0.25 per cent hydrolyzed casein. The results are shown in table 6.

The lowest concentration of Frankonite removed virtually all the riboflavin judging by the disappearance of the pronounced yellowish-green color of the untreated deproteinized milk. *S. liquefaciens* also grows poorly in fuller's earth-treated PFM. Additions of pure vitamin B_1 (gift of the Winthrop Chemical Company), riboflavin, guanine, uracil, and a hydrolysate of yeast nucleic acid—the last suggested by Richardson's (1936)

findings for *Staphylococcus aureus*—to the fuller's earth filtrates did not improve growth.

It was hoped that differences in vitamin requirements might be a convenient supplementary means for differentiating species of streptococci, particularly in the "*viridans*" group. Thirty-five strains of *S. bovis* from Mrs. C. N. Stark's collection were inoculated into deproteinized milk plus casein hydrolysate, yeast extract, and both together. The widest diversity was revealed: some grew in either; others in one but not the other; others only in both together; and still others poorly even in both vitamin sources together. This behaviour was foreshadowed by their growth in litmus milk in which they were maintained where some grew so poorly as not to curdle the milk while others did so in a few hours. Similar results, though with fewer strains, were obtained with *S. salivarius*.

DISCUSSION

All streptococci investigated to date require at least one non-amino-acid growth factor. Its adsorption on fuller's earth and precipitation by mercuric salts are properties of a base. If the theory of the close phylogenetic relation between staphylococci and streptococci be accepted, then Knight's (1937) discovery that *Staphylococcus aureus* needs nicotinic acid or its amide besides vitamin B_1 or its cleavage products, is perhaps applicable to streptococci. The properties of nicotinic acid or of its amide are in keeping with those reported for the hypothetical streptococcus vitamin. Unfortunately, time did not permit trial of these compounds.

The difficulty of concentrating vitamin preparations to the point where their amino-acid content is negligible precludes any sweeping statements as to the number of amino acids that might be required even should one succeed in replacing the casein hydrolysate with known amino acids. No streptococcus was found capable of growing with ammonium salts as a sole source of nitrogen; as previously mentioned growth would occasionally occur in deproteinized milk in the absence of the casein hydrolysate, but came to an abrupt end on serial subculture. Orla-Jensen's con-

clusions as to amino-acid requirements have been criticized on similar grounds by Wood, Anderson and Werkman (1937). Ehrismann and Dramburg (1937) in a study of a strain of *S. pyogenes* mention that cystine was supplied as sulfur source but experimental evidence in support of this was not given in their paper.

A stimulating effect of riboflavin was not observed—a finding opposed to that of Orla-Jensen's.

CONCLUSIONS

By the use of deproteinized skim milk and a casein hydrolysate, amino acid requirements for several species of streptococci may be demonstrated.

Strains of *Streptococcus asalignus*, *Streptococcus bovis* and *Streptococcus mastitidis* were found that required cystine (or cysteine) in addition to at least one more amino acid present in acid-hydrolyzed casein.

Treatment of deproteinized milk with fuller's earth inactivated it for the above streptococci as well as for *Streptococcus liquefaciens*.

Streptococcus bovis requires at least two non-amino-acid factors: one precipitated by mercuric sulfate, the other not.

A great deal of variability in growth requirements was found within the species *Streptococcus bovis* and *Streptococcus salivarius*.

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REFERENCES

Ehrismann, O., and Dramburg, K. 1937 Über die Verwertung von Aminosäuren durch Streptokokken. I. Über die Bedeutung von Aminosäuren fur das Wachstum von Streptokokken. Zeit. Hyg., 119, 623–634.

Evans, Alice C. 1936 Studies on hemolytic streptococci. II. *Streptococcus pyogenes*. Jour. Bact., 31, 611–624.

Freedman, L., and Funk, C. 1922 Nutritional factors in the growth of yeasts and bacteria. I. Vitamines. II. Protein hydrolysates. Jour. Metab. Res., 1, 457–468; 469–480.

Hosoya, S., and Kuroya, M. 1923 Water-soluble vitamine and bacterial growth, with special reference to the chemical and physical properties

of vitamin essential for the growth of hemolytic streptococci. Gov. Inst. Infect. Dis. Tokyo Imp. Univ., 2, 265-304.

KNIGHT, B. C. J. G. 1937 The nutrition of *Staphylococcus aureus*. The activities of nicotinamide, aneurin (vitamin B_1) and related compounds. Biochem. Jour., 31, 966-973.

MUELLER, J. H. 1922 Studies on cultural requirements of bacteria. Jour. Bact., 7, 309-324; 325-338.

OKUDA, Y., AND ZOLLER, H. F. 1921 The relations of hydrogen-ion concentration to the heat coagulation of proteins in Swiss cheese whey. Jour. Ind. Eng. Chem., 13, 515-519.

ORLA-JENSEN, S., OTTE, N. C., AND SNOG-KJAER, A. 1936a Der Vitaminbedarf der Milchsäurebakterien. Centbl. Bakt., II Abt., 94, 434-447.

ORLA-JENSEN, S., OTTE, N. C., AND SNOG-KJAER, A. 1936b Die Stickstoffnährung der Milchsäurebakterien. Centbl. Bakt., II Abt., 94, 460-477.

RICHARDSON, G. M. 1936 The nutrition of *Staphylococcus aureus*. Necessity of uracil for anaerobic growth. Biochem. Jour., 30, 2184-2190.

WOOD, H. G., ANDERSON, A. A., AND WERKMAN, C. H. 1937 Growth-factors for propionic and lactic acid bacteria. Proc. Soc. Exptl. Biol. Med., 36, 217-219.

THE ELECTROPHORETIC MIGRATION VELOCITY OF ESCHERICHIA COLI AFTER CULTIVATION ON MEDIA OF VARIOUS COMPOSITION[1,2]

II. Observations Following Changes in Inorganic Constituents

RALPH P. TITTSLER and GEORGE PACKER BERRY

Department of Bacteriology, University of Rochester, School of Medicine and Dentistry, Rochester, New York

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Although numerous workers, especially Northrop and DeKruif (1922) and Winslow, *et al.* (1923 and 1926), have studied the influence of electrolytes upon the electrophoretic migration velocity of bacteria by washing them free of their metabolic products and suspending them in solutions of the electrolytes, little attention has been given to the effect upon electrophoretic potential of electrolytes incorporated in the culture medium. This is more than a little strange, for it is well known that certain electrolytes, when added to the culture medium, will induce bacterial dissociation or variation which may be recognized readily by alterations in virulence, toxigenicity, agglutinability, *etc.*, alterations which in turn may be accompanied by measurable differences in the electrophoretic migration velocity of the organisms. These considerations suggested that it would be profitable to determine the effect upon the electrophoretic migration velocity of bacteria by varying the electrolyte content of the culture medium. In a previous report (1938) we showed

[1] Based on portions of a thesis presented by Ralph P. Tittsler, June 1, 1933, to the Faculty of the Graduate School of the University of Rochester in partial fulfillment of the requirements for the degree, Doctor of Philosophy.

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that the migration velocity of *Escherichia coli* "remained constant during serial cultivation on a wide variety of culture media differing qualitatively and quantitatively in their organic ingredients." The present paper gives the results of the investigation which was made to ascertain the effect of differences in the inorganic composition of the culture medium upon the electrophoretic potential of *Escherichia coli.*

MATERIALS AND METHODS

Culture media. For the present investigation 29 different culture media were used. In their preparation the ingredients were made to vary both qualitatively and quantitatively. The composition of each medium is recorded in table 1, where each different combination is given a key number for convenience of designation in this report. These numbers begin at 36, numbers from 1 through 35 having been used in a previous paper (1938).

The agar and peptone employed were Difco products, the carbohydrates Pfanstiehl preparations. The sodium, lithium and calcium chlorides, and the sodium phosphates were "Baker's Analyzed Chemicals."

Media numbers 36 to 43, inclusive, were adjusted by adding either hydrochloric acid or sodium hydroxide to give a series of pH values from 5.0 to 8.3.

Procedures. With the exception of the composition of the culture media, the materials and methods, *i.e.*, the sterilization and storage of culture media, the test organism, the inoculation and incubation of cultures, the preparation of bacterial suspensions for electrophoresis, the electrophoresis apparatus and the measurement of the electrophoretic migration velocity, were the same as those described in our previous paper (1938). Thus, only certain points need to be mentioned here.

The test organism, *Escherichia coli,* was cultivated on each of the various media listed in table 1, except numbers 44 to 48, inclusive, for 10 serial transfers at 24-hour intervals. A "stock" series of cultures, carried continuously on standard nutrient agar with transfers every 24 hours, provided a uniform starting point for each experiment with each of the media. To provide the

TABLE 1

Individual formulae of culture media

MEDIUM NUMBER	PER CENT OF		MOLARITY OF			pH
	Agar	Peptone	Sodium chloride	Lithium chloride	Calcium chloride	
36*	2.0	1.0				5.0
37	2.0	1.0				5.5
38	2.0	1.0				6.0
39	2.0	1.0				6.5
40	2.0	1.0				7.1
41	2.0	1.0				7.4
42	2.0	1.0				7.8
43	2.0	1.0				8.3
44–48†	2.0	1.0				6.8–7.1
49‡	2.0	1.0				6.8
50	2.0	1.0	0.016			6.9
51	2.0	1.0	0.031			6.9
52	2.0	1.0	0.062			6.9
53	2.0	1.0	0.125			6.9
54	2.0	1.0	0.250			6.9
55	2.0	1.0	0.500			6.9
56	2.0	1.0		0.031		7.0
57	2.0	1.0		0.062		7.0
58	2.0	1.0		0.125		7.0
59	2.0	1.0		0.250		7.1
60	2.0	1.0			0.008	7.0
61	2.0	1.0			0.016	7.0
62	2.0	1.0			0.031	7.0
63	2.0	1.0			0.062	6.9
64	2.0	1.0			0.125	7.0

* Numbers from 1 through 35 were used in our previous report (1938).

† These media also contained 1.0 per cent of one of the following carbohydrates, glucose, lactose, sucrose, salicin, or mannitol.

‡ This medium also contained 1.5 per cent of NaH_2PO_4 and 1.5 per cent of Na_2HPO_4.

bacteria for observation in the electrophoresis cell, transfers from the "stock" cultures and from the second, fourth, seventh and ninth serial transfers on each medium were made to a slant of the specific medium under investigation. After incubation at 37°C.

for from 12 to 15 hours, the bacteria were removed from the agar, washed three times in double-distilled water, resuspended in double-distilled water, and used for electrophoretic measurements. Mudd's modification of the Northrop-Kunitz microscopic electrophoresis apparatus was used. The migration velocity of 60 or more bacteria in each suspension was determined. The values reported are the average number of seconds required for the bacteria to be moved a distance of 340 microns under the influence of a potential gradient of 3.5 volts per centimeter.

RESULTS

The electrophoretic migration velocity of *Escherichia coli*, when measured under strictly uniform conditions, was found to be constant regardless of qualitative or quantitative differences in the electrolyte composition of the culture medium, with the exception of cultures which were grown on media containing lithium chloride. It was also constant during serial cultivation on all of the various media. Obviously, the migration velocity was not exactly the same for all of the 175 suspensions of bacteria. The differences, however, with the exception just noted, were so small that they must be attributed to experimental error. Furthermore, they did not parallel either the differences in the composition of the culture medium or the progress of serial cultivation. On the other hand, the differences between the velocity of bacteria grown on media containing lithium chloride and that of bacteria cultivated continuously on nutrient agar were slightly greater than the experimental error and, moreover, they paralleled the concentration of the lithium chloride.

The results of the electrophoretic measurements made with bacteria grown on media 36 to 43, inclusive, adjusted to give a series of pH values from 5.0 to 8.3, are recorded in table 2. It is evident that the migration velocity was not affected by differences in the initial pH of the culture medium.

Almost identical results were obtained with bacteria grown without serial passage on peptone agar, number 40, and on peptone agar which contained 1.0 per cent of one of the following carbohydrates, glucose, lactose, sucrose, salicin, and mannitol

(numbers 44 to 48). In fact the greatest difference between the average migration velocity of bacteria grown on any of these carbohydrate media and any average value given in table 2 was less than 5 per cent. The pH values of the carbohydrate media after the growth and removal of the bacteria were as follows: peptone, sucrose and salicin agars, pH 7.7; mannitol agar, pH 6.5; lactose agar, pH 6.1; and glucose agar, pH 5.7.

The migration velocity of bacteria cultivated on a medium containing sodium phosphates, number 49, was the same as that of bacteria grown on media 36 to 48, inclusive.

TABLE 2

Electrophoretic migration time in seconds of bacteria from serial transfers on media of various initial pH values

INITIAL pH OF MEDIUM	SERIAL TRANSFER NUMBER							AVERAGE
	1	1	1	3	6	8	10	
5.0	6.60	6.71	6.79	6.60	6.22	6.35	6.20	6.49
5.5	6.97	6.74	6.74	6.81	6.44	6.47	6.50	6.67
6.0	6.83	6.70		6.88	6.57	6.51	6.18	6.61
6.5	6.89	6.87	6.82	6.82	6.52	6.35	6.31	6.65
7.1	6.85	6.65		6.87	6.68	6.51	6.27	6.64
7.4	6.73	6.87	6.70	6.78	6.35	6.51	6.31	6.61
7.8	6.74	6.58	6.69	6.79		6.59	6.16	6.59
8.3	6.71	6.75	6.75	6.77	6.77	6.43	6.26	6.63
Average..	6.79	6.74	6.75	6.79	6.51	6.46	6.27	6.61

The results obtained with bacteria grown on media containing various concentrations of sodium chloride (table 3) show that the migration velocity was not influenced by the presence of this electrolyte in the culture medium during serial cultivation. It is also evident that amounts of sodium chloride less than 0.5 M did not produce any significant change. The average migration time of bacteria from all of the serial transfers on the medium containing 0.5 M sodium chloride was 0.31 second or 4.5 per cent more than that of bacteria from the control medium, and 0.74 second or 11.4 per cent greater than that of bacteria from the medium containing 0.125 M sodium chloride. The significance of differences of this latter magnitude is difficult to

evaluate, but the findings are probably due to experimental error. We do not feel justified in being dogmatic, however, because the differences are consistent.

TABLE 3

Electrophoretic migration time in seconds of bacteria from serial transfers on media containing various concentrations of sodium chloride

MOLARITY OF NaCl	SERIAL TRANSFER NUMBER				AVERAGE
	1	3	5	9	
0.000	6.72	6.66	6.94	7.32	6.91
0.016	6.76	6.47	6.47	6.85	6.64
0.031	6.71	6.51	6.39	6.62	6.56
0.062	6.53	6.39	6.40	6.81	6.53
0.125	6.55	6.48	6.50	6.39	6.48
0.250	6.63	6.57	6.99	6.76	6.74
0.500	7.05	7.09	7.09	7.64	7.22
Average...	6.71	6.60	6.68	6.91	6.73

TABLE 4

Electrophoretic migration time in seconds of bacteria from serial transfers on media containing various concentrations of lithium chloride

MOLARITY OF LiCl	SERIAL TRANSFER NUMBER				AVERAGE	PER CENT FASTER THAN CONTROL
	1	1	5	8		
0.000	6.63	6.80	6.63	6.80	6.71	
0.031	6.50		6.42	6.80	6.57	2.1
0.062	6.35	6.35	6.22	6.09	6.25	6.8
0.125	5.92	5.91	6.25	6.12	6.05	9.8
0.250		5.86*	5.85*	5.45	5.72	14.7

* This value represents the migration time of the majority of the bacteria, but it was from 5.0 to 5.2 seconds (increased velocity) for approximately 10 or 15 per cent of the cells.

The results of migration velocity measurements made with bacteria which had been grown on media containing various concentrations of lithium chloride are given in table 4. It is evident that no change occurred during serial cultivation, but the values for the bacteria from the media containing 0.250 and 0.125 M lithium chloride are 15 and 10 per cent lower than the

control, and there is a sequence of decreasing values accompanying the increased concentration of lithium chloride in the medium. The differences are so consistent, and of such magnitude in the case of 0.250 and 0.125 M concentrations that they are significant. Although the values reported for the 0.25 M concentration represented the migration time of the majority of the bacteria, it was noted that 10 or 15 per cent of the bacteria were moved much faster. The value for these cells was approximately 5.0 or 5.2 seconds, thus their velocity was about 25 per cent faster than that of bacteria from the control medium. These few data indicate that the electrophoretic migration velocity was increased (lower

TABLE 5

Electrophoretic migration time in seconds of bacteria from serial transfers on media containing various concentrations of calcium chloride

MOLARITY OF $CaCl_2$	BACTERIA WASHED 3 TIMES						BACTERIA WASHED 4 TIMES			
	Serial transfer					Average	Serial transfer			Average
	1	3	5	7	9		3	7	9	
0.000	6.68	6.91	6.99	7.18	6.87	6.93	6.95	6.98	7.26	7.06
0.008	6.41	6.82	6.89	6.65	6.74	6.70	6.87	6.94		6.90
0.016	6.59	6.53	6.83	6.67	6.48	6.62	6 87	6.64	7.16	6.89
0.031	6.34	7.24	6.90	6.99	7.48	7.19	6.52	6.94	7.06	6.84
0.062		8.60	6.72	7.68	8.25	7.81	6.79	7.99	7.06	7.28*
0.125		7.07	7.40	6.45	7.51	7.11		6.94	6.62	6.78

* This average was greatly affected by one individual determination (7.99).

values) by the presence of lithium chloride in the culture medium, but further experiments are required to establish this point.

The first series of experiments which was made with bacteria grown on media containing various concentrations of calcium chloride yielded irregular results with the higher concentrations of the salt. This suggested that threé washings were perhaps insufficient to remove completely the calcium chloride carried over with the bacteria from the culture medium. Therefore, the experiments were repeated using two sets of bacterial suspensions, one of which was washed three times, as usual, and the other four times. To prepare these suspensions, the thrice-washed bacteria were divided into two portions, one for additional

washing and the other for immediate electrophoresis. The results are recorded in table 5. Again, the values for thrice-washed bacteria from 0.031, 0.062 and 0.125 M calcium chloride agars were irregular. In each instance, except one, the values for bacteria which were washed four times were practically identical, regardless of the concentration of calcium chloride in the culture medium. It appears, therefore, that the irregular results obtained with some of the thrice-washed bacteria were due to the incomplete removal of calcium chloride carried over from the culture medium. The migration velocity did not change during serial cultivation in either experiment.

DISCUSSION

To determine whether or not the electrophoretic potential of bacteria is irreversibly altered by variations in the composition of the culture medium, it is necessary to eliminate factors other than changes in the bacteria themselves which influence the electrophoretic migration velocity and to measure the rate of migration under strictly uniform conditions. It is especially necessary to eliminate completely any electrolyte carried over with the bacteria from the culture medium, because even traces in the suspending medium will produce enormous changes in the migration velocity. The procedure employed in this investigation, and described in our previous paper (1938), was designed to meet these requirements.

The results obtained serve to indicate that the presence of certain electrolytes in the nutrient medium does not produce a permanent change in the electrophoretic potential of bacteria. This is certainly true for media adjusted to various initial pH values or altered in pH during the fermentation of carbohydrates, and for media containing either sodium phosphates or sodium chloride.

In view of the general uniformity of the values obtained with bacteria grown on media containing various concentrations of calcium chloride and washed four times, it appears that the *only* conclusion which can be reached at present is that the electrophoretic potential was not permanently affected. This con-

clusion assumes, of course, that the irregular velocities obtained in some instances with the thrice-washed bacteria were due to the incomplete removal of calcium chloride during washing. Pedlow and Lisse (1936) reported variations as great as 33 per cent in the migration velocity of bacteria grown in a medium containing 0.1 M calcium chloride and washed once. Their data also show clearly the effect of repeated washing on the migration velocity of bacteria grown in a medium containing 0.01 M calcium chloride. The velocity of bacteria washed three times was 25 per cent greater than that of bacteria washed once, and 125 per cent greater than that of bacteria removed from the nutrient medium and suspended in distilled water. The velocity increased progressively with the three washings and remained constant thereafter. When washed three times, the velocities of bacteria grown in the presence of either 0.01 M calcium chloride or 0.5 M sodium sulphate were practically the same as that of bacteria from the control medium. Thus, they concluded that "the salts had produced no irreversible change in the nature of the bacterial surface." From the results of another portion of their investigation, they concluded that "the electrophoretic mobility can be increased or decreased by the presence of certain salts in the growth medium." In this instance, however, the bacteria were washed only once. We believe, therefore, that the changes which they reported in the migration velocity were caused by the presence of electrolytes carried over with the bacteria.

As pointed out previously, the increased migration velocity recorded in the present investigation for bacteria grown in the presence of lithium chloride suggests that the bacteria were irreversibly changed. The data, however, are not sufficient to warrant a definite conclusion at the present time, but suggest that further investigations might profitably be carried out.

When cations, especially hydrogen ions, are added to suspensions of washed bacteria, a marked decrease in the migration velocity of the cells follows, sometimes resulting in a condition of isopotential or even complete reversal in the electrophoretic potential of the bacteria. Since the presence of certain electrolytes in the culture medium has been found to produce no

change in the migration velocity of thoroughly washed bacteria, the depressant effect of cations on the electrophoretic potential must be caused either by changes in the suspending medium or by a reversible influence upon the cell itself. Hence the question, whether the depressant effect of all cations be abolished upon their removal from the suspending medium or whether an irreversible change in the cell had been produced. If the latter possibility be true, it may be asked further, whether the change be limited to the surface of the cell, or be within the cell. If the depressant effect of all cations be reversible, it follows that their presence in the nutrient medium does not change the electrophoretic potential, provided that they are completely removed from the cells and the suspending medium. It may be true, however, that the effect on the electrophoretic potential of some cations is reversible, while that of others is irreversible. The results of this investigation indicate that the effects of hydrogen, sodium, and calcium ions are reversible, while those of lithium may be irreversible. Pearce, *et al.* (1935) could detect no permanent effects from low concentrations in the nutrient medium of sodium chloride, calcium chloride, aluminum chloride, sodium phosphate, or sodium sulphate. Pedlow and Lisse (1936) found the effects of sodium sulphate and calcium chloride to be reversible.

SUMMARY

The electrophoretic velocity of *Escherichia coli*, when measured under strictly uniform conditions, remained constant during serial cultivation on a variety of nutrient media differing qualitatively and quantitatively in their inorganic ingredients, with the probable exception of cultures which were grown on media containing lithium chloride. Further observations are needed to determine definitely the effect of this salt.

The constant electrophoretic velocity of the bacteria is considered to constitute strong evidence, but not necessarily proof, that the physico-chemical composition of cells was not permanently changed by variations in the culture medium, and that the effects of the cations studied, other than lithium, upon the electrophoretic potential of bacteria are reversible.

REFERENCES

Northrop, J. H., and DeKruif, P. H. 1922 The stability of bacterial suspensions. II. The agglutination of the bacillus of rabbit septicemia and of *Bacillus typhosus* by electrolytes. Jour. Gen. Physiol., **4**, 639–655.

Pearce, G. W., Lisse, M. W., and Tittsler, R. P. 1935 Influence of electrolytes added to growth medium on electrophoretic potential of *Escherichia coli*. Proc. Soc. Exp. Biol. and Med., **32**, 1572–1573.

Pedlow, J. T., and Lisse, M. W. 1936 The effect of electrolytes present in the growth media upon the electrophoretic mobility of *Escherichia coli*. Jour. Bact., **31**, 235–244.

Tittsler, R. P., and Berry, G. P. 1938 The electrophoretic migration velocity of *Escherichia coli* after cultivation on media of various composition. I. Observations following changes in organic constituents. Jour. Bact., **35**, 213–222.

Winslow, C.-E. A., Falk, I. S., and Caulfield, M. F. 1923 Electrophoresis of bacteria as influenced by hydrogen ion concentration and the presence of sodium and calcium salts. Jour. Gen. Physiol., **6**, 177–200.

Winslow, C.-E. A., and Fleeson, E. H. 1926 The influence of electrolytes upon the electrophoretic migration of bacteria and of yeast cells. Jour. Gen. Physiol., **8**, 195–214.

Winslow, C.-E. A., and Upton, M. F. 1926 The electrophoretic migration of various types of vegetable cells. Jour. Bact., **11**, 367–392.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

ILLINOIS BRANCH

WINTER MEETING, CHICAGO, JANUARY 21, 1938

INFECTIONS OF THE SKIN. *Cleveland White.* Northwestern University, Chicago.

BACTERIOLOGICAL ASPECTS OF THE CHEESE INDUSTRY. *J. B. Stine,* Kraft-Phoenix Cheese Corporation, Chicago.

EXPERIMENTAL PULMONARY TUBERCULOSIS IN THE DOG.* *Moore A. Mills, Francis D. Gunn and E. E. Barth,* Departments of Bacteriology, Pathology and Radiology, Northwestern University Medical School, Chicago.

The comparative resistance to tuberculous infection (human and bovine strains) of various experimental animals and man was discussed. Of all common laboratory animals the dog is considered to be the most similar to man in relative resistance and in the type of lesion which results. Pulmonary lesions in dogs were produced by intra-bronchial instillation of suspensions in mucin of living virulent human and bovine strains of tubercle bacilli by means of a bronchoscope. The dosage employed varied from 0.0075 to 0.5 mgm. per kilo. Complete clinical studies including blood counts, sedimentation rates, tuberculin tests for cutaneous sensitivity and routine X-ray examinations were made upon each dog.

The appearance of resulting lesions depends more upon the individual susceptibility of the animal than upon the dosage. Lesions varying from proliferative epitheloid masses through caseation and cavity formation were found. Healing, including obliteration of cavities, occurred in the more resistant animals. Skin sensitivity to tuberculin in cases of active infection is demonstrable by the 5th or 7th day and persists for as long as the experiments have lasted, becoming negative only in cases with rapidly progressing or far advanced tuberculosis.

* Aided by a grant from the Medical Research Council of the American Medical Association.

SOME ELEMENTS OF MOLD CONTROL IN THE FOOD INDUSTRY. *M. L. Laing, Research Laboratories, Armour and Company, Chicago.*

STUDIES ON ANAEROBIOSIS

I. The Nature of the Inhibition of Growth of Cyanide-Treated E. coli by Reversible Oxidation-Reduction Systems[1]

R. H. BROH-KAHN and I. ARTHUR MIRSKY

Department of Metabolism and Endocrinology, Institute for Medical Research, The Jewish Hospital, Cincinnati, Ohio

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The mechanism whereby oxygen inhibits the growth of obligate anaerobes has been the subject of much interest. Three outstanding explanations have been offered:

(1) That oxygen is directly lethal to the cell (Pasteur (1861)).

(2) That hydrogen peroxide, as the first reduction product of oxygen, is lethal to the cell lacking catalase (M'Leod and Gordon (1923, a, b)).

(3) That the growth of anaerobes is dependent upon the presence of a low oxidation-reduction potential, the attainment of which is prevented by oxygen (Quastel and Stephenson (1926)).

The observation that growth ensues when anaerobes are transferred from an oxygen-rich medium to an anaerobic one eliminates the first hypothesis (Quastel and Stephenson). The concept presented by M'Leod and Gordon receives some support from the fact that most obligate anaerobes lack catalase and hence are very sensitive to hydrogen peroxide. In accord with this are the observations on the "suicide" of pneumococci grown aerobically, which, in view of their lack of catalase, can be attributed to the accumulation of peroxide. On the other hand, the hypothesis does not account for the anaerobes which do not lack catalase. Furthermore, it implies that anaerobes consume oxygen in order to produce peroxide, a phenomenon which Fujita

[1] Aided by the David May Memorial Fund.

and Kodama (1934) could not observe. Finally, direct evidence of peroxide formation by obligate anaerobes exposed to oxygen has never been offered.

The hypothesis of Quastel and Stephenson is based on the observations of Clark (1934) and others that cells living under anaerobic conditions produce more negative oxidation-reduction potentials than those in an aerobic environment. Quastel's concept would appear to imply that negative potentials precede rather than result from growth and that oxygen and peroxide prevent the establishment of reducing conditions sufficiently high to permit growth. This hypothesis is open to criticism on several counts. In the first place, it is obvious that the free energy released by cellular growth is primarily determined by the constituents of the culture medium; the cells influence this factor only in so far as they determine which constituents of the medium are utilized or by what paths they are consumed. Accordingly, the potentials observed are characteristic of the medium rather than of the organism. This is illustrated by comparing the very different potentials of *Escherichia coli* actively fermenting glucose and of the same organism growing in an asparagine medium. Likewise, as Kluyver and Hoogerheide (1936) have demonstrated, two different organisms growing in the same medium, will produce the same overall potentials, regardless of the nature of their respiratory mechanisms, if they utilize the same substances. It appears to be more reasonable to assume that the low potentials commonly observed in anaerobic cultures are due to the interactions between the constituents of the medium catalyzed by *growing* cells. In view of Stickland's fundamental observations (1934, 1935, a, b, c) that certain obligate anaerobes obtain their energy from coupled reactions between amino acids, it can be estimated that the potentials produced from such reactions can account for the low values observed. Furthermore, contrary to Quastel's hypothesis is the observation of Dack and Burrows (1935) that certain non-sporulating obligate anaerobes produce potentials positive in many cases to those observed in actively growing cultures of aerobes. Hence it is probable that obligate anaerobes neither

require nor produce very low potentials save through the chain of reactions available to them for energy, either through the enzyme systems they possess, or through the actual culture medium offered them.

From the above considerations it is obvious that the accumulation of more data which may lead to a better understanding of the general problem of anaerobiosis is warranted. We began our studies with an investigation of the anaerobic mechanism of facultative anaerobes as compared with that of obligate anaerobes. Novy (1925) postulated that this was identical in both types of anaerobes, the only difference being the presence of an aerobic system in the facultative anaerobes. Accordingly, it might be anticipated that the destruction of the aerobic system of a facultative anaerobe would transform the latter into an obligate anaerobe. Our studies reveal that such is not the case and that fundamental differences exist between the anaerobic systems of facultative and obligate anaerobes with respect to their response to oxygen and to changes in the oxidation-reduction potentials of their environment.

EXPERIMENTAL

An intestinal strain of *Escherichia coli*, kindly furnished by T. L. Snyder of the Department of Bacteriology, University Cincinnati, College of Medicine, was employed in all the experiments and maintained on the usual laboratory media. Inoculations were always made with two drops of a fresh, eight-hour culture of the organism in nutrient broth. Anaerobic incubation was secured either with alkaline pyrogallate or by an anaerobic jar. Growth was estimated nephelometrically with the aid of arbitrary standards and recorded with the usual conventions of plus and minus signs. It is recognized that such estimations are rough, but the great differences in turbidity between the different standards and the fact that only large differences in growth are reported as significant have led us to adopt this method.

Where "nutrient" media are designated, the usual meat-extract peptone base was employed and made up with water to

such a concentration that the final additions of material would give the customary dilution. Such concentrated media were tubed in definite volumes and sterilized. Just prior to inoculations, the various other materials were added by sterile pipettes so that each tube contained known and comparable amounts of material.

To destroy the aerobic mechanism of *E. coli*, freshly prepared solutions of M or 0.1 M KCN carefully brought to pH 7.6 by molar KH_2PO_4 were used. In early experiments, such solutions were sterilized by filtration, but this method was discarded when it became apparent that fresh cyanide solutions never produced growth when added to sterile media. In order to replace the respiratory system of cyanide-poisoned *E. coli*, solutions of various oxidation-reduction systems were utilized. These were so prepared from analyzed samples that the dilutions recorded are true values of dye content.[2]

In investigating the influence of adding a respiratory system to the cyanide-treated *E. coli* it became necessary to study the accumulation of H_2O_2 in the cultures. Hydrogen peroxide was detected by paper impregnated with a concentration solution of KI in fresh starch solution. Very little practice was required to estimate 0.0003 per cent H_2O_2 with certainty, as was demonstrated with known dilutions of peroxide. When cultures were examined for peroxide, kaolin was added to remove the dyes, and the acidified filtrates tested with the starch-iodide paper. In all positive tests, the paper not only turned brownish-blue, but the liquid itself rapidly became blue from diffusion from the paper.

All experiments were performed in duplicate and repeated at least once. For the sake of clarity, they may be best considered under various headings.

I. The concentration of cyanide necessary to inhibit growth

In order to determine that concentration of HCN which would inhibit growth under the conditions employed in this investiga-

[2] We are indebted to the National Aniline and Chemical Company, Inc. for generous supplies of analyzed samples of Nile blue A, Phenosafranine and Briliant Cresyl blue.

tion, a series of tubes containing the same amount of concentrated 1 per cent glucose nutrient broth was set up and to these were added identical volumes of progressive dilutions of KCN. They were immediately inoculated with *E. coli* and incubated aerobically and anaerobically. In table 1 are found the results of this experiment. That the concentrations of cyanide found necessary to inhibit growth are slightly higher than those reported by Burnet (1927), who employed tryptic-digest glucose agar

TABLE 1

Effect of varying concentrations of cyanide on the growth of E. coli in glucose nutrient broth

	MOLARITY KCN						
	0	M/1000	M/800	M/600	M/500	M/400	M/300
Aerobic incubation:							
Growth 16 hours	++++	++++	++++	++++	+++	++	++
Growth 48 hours	++++	++++	++++	++++	++++	++++	++++
Anaerobic incubation:							
Growth 16 hours	++++	++++	++++	++++	++++	+++	+++
Growth 48 hours	++++	++++	++++	++++	++++	++++	++++

	MOLARITY KCN						
	M/200	M/100	M/80	M/60	M/40	M/20	M/10
Aerobic incubation:							
Growth 16 hours	++	++	++	++	±	–	–
Growth 48 hours	++++	++++	+++	++	+++	–	–
Anaerobic incubation:							
Growth 16 hours	++	++	++	++	±	–	–
Growth 48 hours	++++	++++	++	++	++	–	–

plates, is not surprising, considering the well known variations between liquid and solid media.

M/600 KCN is known to produce complete paralysis of the iron-containing respiratory catalysts in the concentrations in which they exist in cell aggregates and concentrations of this order have been extensively employed in respiration experiments. However, when dealing with growth phenomena, larger quantities must be employed. Apparently, the cyanide is extensively volatilized during incubation and the concentrations rapidly

decrease from their initial values. Moreover, as will be demonstrated, growth proceeds even though oxygen consumption has ceased.[3] It is possible too that the extensive multiplication, even under these "anoxytrophic" conditions, may synthesize enough respiratory catalyst to overcome the concentrations of cyanide initially present.

In order, then, to make certain that growth occurs under conditions in which oxygen can not be utilized by the cells M/100 KCN was adopted for routine usage in these experiments. It suffices, as will be demonstrated, to prevent oxytrophic growth for at least twenty-four hours and completely inhibits catalase action, but does not greatly affect anaerobic growth. Larger concentrations of cyanide produce actual irreversible damage to the cell. Buchanan (1926) has discussed the various cellular factors beside respiration catalysis that may be affected.

II. The "anoxytrophic" growth of cyanide-treated E. coli in the presence of oxygen

Burnet (1927) concluded that cyanide prevented the growth of all the organisms investigated by him because it poisoned the mechanism by which the cells destroy hydrogen peroxide. In many cases, such could not be the case. For example, according to the data of Fujita and Kodama (1934), the oxygen consumption of *E. coli* would be completely inhibited by 0.1 per cent KCN. For peroxide to be formed, oxygen must first be reduced. (See discussion.) It is obvious then, that although KCN does inhibit catalase action, it must also, in the case of the large group of organisms, whose respiration is inhibited by it, prevent the formation of H_2O_2. We are rather inclined to attribute complete

[3] The terms *anaerobic* and *aerobic* are usually reserved to indicate growth in the absence or presence of air. In order to subscribe to general usage and, at the same time, to prevent confusion, we have chosen the term *anoxytrophic* to indicate growth under conditions in which oxygen, whether present or not, is not *utilized* by the cells. Similarly, *oxytrophic* will be used to describe growth during which molecular oxygen is employed by the cell to obtain energy. Thus anoxytrophic growth may occur under aerobic conditions.

growth inhibition by high concentrations of cyanide to actual irreversible cell damage. In the case of an organism like the staphylococcus which possesses a cyanide-insensitive respiratory mechanism (Fujita and Kodama, 1934) and which is capable of peroxide formation even in the presence of cyanide, Burnet's explanation may be correct. Experimental evidence on this point is reported in a later section.

The fact that growth readily occurred in the presence of M/100 cyanide was interpreted as an inhibition of oxytrophic growth without evidence of great damage to anoxytrophic proliferation. In order to demonstrate that the latter type of growth actually does proceed while oxytrophic multiplication is prevented, the

TABLE 2

Effect of M/100 KCN on the growth of E. coli in various synthetic media—24-hour incubation

MEDIUM	LACTATE	LACTATE + KCN	LACTATE-FUMARATE	LACTATE-FUMARATE + KCN	LACTATE-NITRATE	LACTATE-NITRATE + KCN
Aerobic incubation........	++++	–	++++	++	++++	–
Anaerobic incubation......	++++	–	+++	++	++++	–

following experiments were performed. Quastel, Stephenson and Whetham (1925) undertook an extensive investigation into the conditions necessary for the anaerobic growth of *E. coli*. As a result of their researches, they developed a synthetic medium of the following composition: 0.4 gram $(NH_4)_2HPO_4$, 0.1 gram KH_2PO_4, 0.07 gram $MgSO_4 \cdot 7H_2O$, Trace $FeSO_4$, M/10 lactate, 90 cc. distilled water, adjust to pH 7, which would support aerobic but not anaerobic growth of *E. coli*. If, however, 0.1 M fumarate were added to the above, the fumarate-lactate medium would afford good anaerobic growth. If it is true, then, that M/100 KCN completely inhibits oxytrophic growth but allows anoxytrophic growth to proceed, when sufficient cyanide (0.5 cc. of M/10 KCN per 4.5 cc. of medium) is added, the lactate-inorganic salt medium should not support growth, whereas the lactate-fumarate synthetic medium should do so. Table 2 shows

that such is the case.[4] Furthermore, KCN, as will be reported at more length in another paper, inhibits the function of the enzyme concerned with the "activation" of nitrate and thus renders a nitrate-lactate-inorganic-salt medium incapable of supporting anaerobic growth.

III. The influence of substituting various oxidation-reduction systems for the respiratory system destroyed by cyanide

Since *E. coli* differs from some other organisms in not possessing a cyanide-insensitive mechanism for oxygen consumption (Fujita and Kodama), it offers an excellent test object for the effect of oxygen *availability* and of hydrogen peroxide accumulation on growth. For this purpose, it is only necessary to substitute for the inactivated respiratory mechanism, one capable of functioning in the presence of cyanide. Fleisch (1924) and Barron and Hamburger (1932) among others have demonstrated that certain dyes are capable of substituting for a cyanide-poisoned respiratory catalyst. If, then, to a medium containing cyanide, methylene blue is added, the effect of oxygen consumption on such a preparation can be tested. In table 3 it is shown that the addition of methylene blue completely inhibits growth in air. Quastel and Wheatley (1931) have convincingly demonstrated the inhibitions displayed by various dyes towards certain enzyme systems. Before it is possible to attribute this inhibitory activity of the dye to its action in restoring respiration, it is necessary to demonstrate its non-specific action, i.e., to prove that it is acting catalytically. For this purpose, the following experiments were designed. If methylene blue is inhibiting growth because of a restoration of oxygen consumption its action should be observed in concentrations similar to those found necessary to catalyze oxygen consumption and should disappear when more diluted. Table 3 shows that extremely small quantities are required for

[4] Anaerobic growth in such media is rather poor. It is necessary as Quastel *et al.* point out, to use heavy inocula to obtain consistent results. For similar reasons, it is best to employ M/400 KCN and large inocula in such experiments although, occasionally, as table 2 demonstrates, good growth may be secured by employing M/100 KCN. M/400 cyanide will completely suppress aerobic growth in the lactate-inorganic medium.

this action, far below those reported as actually inhibitive *per se.* Moreover, if the inhibition were due to a specific poisoning by the dye system, it should persist under anaerobic conditions. If, however, the effect is due solely to its ability to promote respiration, *E. coli* should grow in the presence of cyanide plus dye under anaerobic conditions where no oxygen is available. In the presence of cyanide, even 10^{-6} M (table 3) methylene blue exerts some measure of inhibition under anaerobic conditions. This retardation of growth is undoubtedly due to a specific poisoning by the dye. But comparing the degree of inhibition by 10^{-5} M

TABLE 3

Effect of methylene blue on the growth of E. coli in the presence of cyanide in glucose broth

Molarity Methylene Blue.	0	10^{-4}	0	10^{-4}	5×10^{-5}	2×10^{-5}	10^{-5}	5×10^{-6}	2×10^{-6}	10^{-6}
Molarity KCN.	0	0	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}
Aerobic incubation:										
Growth 24 hours	++++	++++	++	−	−	−	−	+	+	+
Growth 48 hours	++++	++++	++++	−	−	−	−	++	++	+++
Anaerobic incubation:										
Growth 24 hours	++++	++++	++	++	++	++	+++	++	++	++
Growth 48 hours	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

methylene blue under aerobic and anaerobic conditions, we feel justified in concluding that the complete cessation of growth under aerobic conditions is not due to a specific poisoning. It appears important to emphasize that the so-called non-inhibition under anaerobiosis is one of degree only.

To complete the chain of evidence that restored oxygen consumption is solely responsible for the observations, it should be possible to demonstrate the same effect with any reversible oxidation-reduction system provided its potential is such that:

(a) The oxidant can be reduced by the negative substrate.

(b) The reductant can be auto-oxidized by molecular oxygen.

TABLE 4

Effect of various oxidation-reduction systems on the growth of E. coli in the presence of cyanide in glucose broth

Concentration of O-R systems is 10^{-4} M. Concentration of KCN is 10^{-2} M. Twenty-four hour incubation period.

CONSTITUENTS ADDED TO BASAL MEDIUM	E'_0 AT pH 7 AND 30°C. OF SYSTEM	AEROBIC INCUBATION		ANAEROBIC INCUBATION	
		Growth	Dye color	Growth	Dye color
	volts				
....		++++		++++	
KCN		++		++	
Neutral red	−0.325	++++	++++	++++	++++
Neutral red + KCN		±	++++	++	++++
Phenosafranine	−0.252	++++	++++	++++	++++
Phenosafranine + KCN		−	++++	++	++++
Nile blue	−0.122	++++	++	++++	±
Nile blue + KCN		−	++++	++	+
Methylene blue	0.011	++++	++	++++	−
Methylene blue + KCN		±	++	++	−
Brilliant cresyl blue	0.047	++++	±	++++	−
Brilliant cresyl blue + KCN		+	++	++	−
1-naphthol-2-sulfonate indophenol	0.123	++++	−	++++	−
1-naphthol-2-sulfonate indophenol + KCN		−	++++	+	−
o-cresol-2,6-dichloro indophenol	0.181	++++	−	++++	−
o-cresol-2,6-dichloro indophenol + KCN		++	−	++	−
$K_3Fe(CN)_6$	0.43	++++		+++	
$K_3Fe(CN)_6$ + KCN		+++		++	
$FeCl_3$	?	++++		++++	
$FeCl_3$ + KCN		+++		++	

To test this possibility, a series of tubes of sterile 1 per cent glucose nutrient broth were set up and a series of oxidation-reduction systems added to some. To half of these KCN was

added. Control tubes containing medium alone and medium + KCN were used. All were inoculated with *E. coli* and half were incubated aerobically and half anaerobically. The results of this experiment are given in table 4 and demonstrate that all dye systems up to a given potential (E'_0(pH 7) = 0.123v) markedly inhibit growth in the presence of KCN under aerobic, but not to nearly the same extent under anaerobic, conditions. The positive systems fail to inhibit because, although they are reducible, the reductants are not rapidly oxidized by air because of their high potential. (For a discussion of the possible relationship between potential and kinetics of oxidation, see Clark (1934).)

It is rather interesting that even the dyes of lowest potential inhibited. Neither neutral red nor phenosafranine were decolorized even anaerobically. However, although the potential of the glucose system has not been worked out as yet, it is known to lie in the neighborhood of the hydrogen electrode and the sugar in the presence of bacteria should partially, at least, reduce the dyes. These partially reduced dyes may be reoxidized so rapidly that they may be as efficient respiratory catalysts as those completely reduced.

Since dyes negative enough not to be reduced by the glucose system were not available, the attempt was made to test the effects of the series of oxidation-reduction systems on a medium incapable of reducing the low dyes. For this purpose, both lactate-fumarate-nutrient medium and lactate-$K_3Fe(CN)_6$-nutrient medium were employed. The potentials of these culture substrates are high enough not to reduce to any extent the lowest dyes. These results are given in table 5 and demonstrate that, in such media, neutral red and phenosafranine did not inhibit growth completely. The reactions involved in cellular syntheses following the initial oxidation of lactate to pyruvate by fumarate and ferricyanide are so little known that it is impossible to calculate the reduction potentials. Apparently, however, the overall oxidation-reduction continuum is high enough not to reduce the low potential dyes.

TABLE 5

Effect of various oxidation-reduction systems on the growth of E. coli in the presence of cyanide in lactate-fumarate and lactate-ferricyanide media

Same conditions as in table 4

CONSTITUENTS ADDED TO BASAL MEDIUM	E'_0 (pH 7) AND 30°C. OF SYSTEM	LACTATE-FUMARATE MEDIUM				LACTATE-FERRICYANIDE MEDIUM			
		Aerobic		Anaerobic		Aerobic		Anaerobic	
		Growth	Dye color	Growth	Dye color	Growth	Dye color	Growth	Dye color
	volts								
....		++++		+++		+++		++	
KCN		+++		+++		++		++	
Neutral red	−0.325	++++	++++	+++	++++	++	++++	++	++++
Neutral red + KCN		++	++++	++	++++	+±	++++	++	++++
Phenosafranine	−0.252	+++	++++	+++	++++	+±	++++	++	++++
Phenosafranine + KCN		+	++++	++	++++	+	++++	+±	++++
Nile blue	−0.122	+++	++++	++	±	+±	+++	+±	++
Nile blue + KCN		−	++++	++	−	−	++++	+±	+
Methylene blue	0.011	+++	+	+++	−	+±	++	++	±
Methylene blue + KCN		±	++	++	−	±	++	++	±
Brilliant cresyl blue	0.047	+++	+	+++	−	+	+	++	−
Brilliant cresyl blue + KCN		+	++	+++	−	±	++++	++	−
1-naphthol-2-sulfonate indophenol	0.123	+++	−	+++	−	+++	−	++	−
1-naphthol-2-sulfonate indophenol + KCN		−	++++	±	−	−	++++	+	−
o-cresol-2,6-dichloroindophenol	0.181	++++	−	+++	−	++	−	+±	−
o-cresol-2,6-dichloroindophenol + KCN		+++	−	+++	−	++	−	+±	−
$K_3Fe(CN)_6$	0.43	++++		+++					
$K_3Fe(CN)_6$ + KCN		+++		++					
$FeCl_3$	?	++++		++++		+++		++	
$FeCl_3$ + KCN		+++		+++		++		++	

IV. The mechanism whereby oxygen inhibits the growth of cyanide-treated E. coli in the presence of various oxidation-reduction systems

Since it has been demonstrated that oxygen consumption is definitely inhibitory to such preparations, the question arises as to whether it is oxygen *per se* or some other factor which is responsible. It has long been known that the primary product of the air-oxidation of certain reduced dyes is H_2O_2. Since *E. coli* in the presence of cyanide is incapable of destroying that peroxide, this substance should be present in the cultures where dyes + KCN inhibited growth. To test this, a series of tubes containing methylene blue + KCN and the proper controls were

TABLE 6

Accumulation of hydrogen peroxide by E. coli in the presence of methylene blue and cyanide in 15-hour cultures in glucose broth

KCN is 10^{-2} M. Methylene blue, 10^{-4} M

CONSTITUENTS ADDED TO BASAL MEDIUM	AEROBIC INCUBATION		ANAEROBIC INCUBATION	
	Growth	Peroxide test	Growth	Peroxide test
....	+++	−	+++	−
KCN	++	−	++	−
Methylene blue	+++	−	+++	−
Methylene blue + KCN	−	+	++	−

inoculated and incubated both aerobically and anaerobically for 15 hours. At the end of that period, they were examined for growth and the presence of H_2O_2. The results in table 6 demonstrate that H_2O_2 could be detected with certainty only in the tubes containing dye + KCN and incubated aerobically. Estimates based on comparisons with known quantities gave amounts of peroxide ranging between 0.0006 and 0.0003 per cent. Such concentrations of peroxide are definitely below those reported by M'Leod and Gordon (1923a) as capable of inhibiting the growth of even the most peroxide-sensitive organisms. In order to ascertain whether or not they are actually capable of inhibiting growth, a series of sterile 1 per cent glucose nutrient broth tubes was prepared and to half of these KCN was added to give a

final concentration of M/100. To both sets of tubes, progressive dilutions of H_2O_2 were added to give concentrations ranging from one down to 0.0005 per cent. These dilutions had been *freshly* prepared from the stock "30 per cent" peroxide which actually contained 25.13 per cent peroxide by iodimetric analysis. Both sets of tubes were inoculated with *E. coli* and incubated aerobically. The results in table 7 indicate that 0.005 per cent peroxide inhibited whereas 0.0025 per cent failed to inhibit growth in the plain medium[5] whereas all dilutions inhibited in the presence of cyanide. This extreme sensitivity to peroxide is difficult to understand. It is possible that the use of different

TABLE 7

Effect of hydrogen peroxide on the growth of E. coli in glucose broth—48-hour aerobic incubation

	PER CENT H_2O_2 IN MEDIUM											
	1	0.1	0.05	0.025	0.02	0.0125	0.01	0.005	0.0025	0.002	0.001	0.0005
Plain medium	−	−	−	−	−	−	−	−	++	+++	++++	++++
Transfer*	−	−	−	−	+	++	+++	+++	++++	++++	++++	++++
Medium + 10^{-2} M KCN	−	−	−	−	−	−	−	−	−	−	−	−
Transfer*	−	−	−	+	+	++	+++	+++	++++	++++	++++	++++

* Two drops of corresponding culture inoculated at end of 48 hours into fresh medium (without cyanide or peroxide) and incubated 24 hours.

media and size of inoculum may partially explain the much lower values than those found necessary by M'Leod and Gordon and Quastel and Stephenson (1926) to inhibit even obligate anaerobes. These results have been so often repeated, however, that there can be little doubt as to their accuracy or as to the fact that *E. coli* poisoned by cyanide is extremely sensitive to H_2O_2 and that the amounts of peroxide found in aerobic cultures containing dyes + KCN are adequate to explain the inhibition of

[5] It is of interest to remark that these low concentrations of H_2O_2 transform the customary "smooth" growth of *E. coli* into an extremely "rough" form. In broth, the growth is heavy, granular and confined entirely to a thick deposit on the bottom of the tube with absolutely no opacity in the rest of the tube. This observation coincides with the finding of Todd (1930) concerning the transformation of hemolytic streptococci from "glossy" to "matt attenuated" strains by peroxide.

growth. The transfers to fresh medium (table 7) further confirm the contention of Quastel and Stephenson that neither oxygen nor H_2O_2 below certain concentrations is actually lethal but merely antiseptic.

DISCUSSION

E. coli in the presence of cyanide, is strictly anoxytrophic in its metabolism because of its complete inability to utilize atmospheric oxygen for energy. It differs from an obligate anaerobe insofar as it can grow as well in the presence as in the absence of air. On the other hand, when the organism is placed in a medium containing HCN plus a reversible dye system of suitable potential, it can no longer tolerate molecular oxygen and, in this respect, is a model of an obligate anaerobe.

This intolerance for oxygen in the presence of a respiratory system but in the absence of catalase was attributed to the formation of H_2O_2 due to the air oxidation of leuco dye. Such an argument appears to be valid inasmuch as oxygen consumption *per se* has no effect on the organism in the presence of catalase (i.e., absence of cyanide). The reversible dye systems act, as Barron and Hoffman have demonstrated (1930) as true respiratory catalysts. In an intact cell as Keilin and others have adduced, respiration may proceed in the following fashion:

$$(1)\quad \text{Substrate} + 2Fe^{+++} \underset{\text{dehydrogenase}}{\rightleftarrows} \text{oxidized substrate} + 2Fe^{++} + 2H^{+}$$

$$(A)\quad (2)\quad 2Fe^{++} + 2H^{+} + O_2 \underset{\text{inodphenoloxidase}}{\rightleftarrows} 2Fe^{+++} + H_2O_2$$

$$(3)\quad H_2O_2 \underset{\text{catalase}}{\rightleftarrows} H_2O + 1/2\, O_2$$

The cyanide sensitivity of this scheme is due solely to the fact that the mechanism (inodphenoloxidase) responsible for the reoxidation of reduced cytochrome is inhibited by cyanides. In their presence, scheme (1) can proceed, but not scheme (2). Inasmuch as cytochrome is present in small amounts only and

can oxidize only equimolecular quantities of substrate, the effect of a cessation of (2) will be followed by a cessation of (1).

No mechanism is present in cyanide-treated *E. coli* for rendering molecular oxygen available to the cell. If, however, there is introduced a substance capable of reduction by the enzyme system and of reoxidation by oxygen, even in the presence of KCN, oxygen utilization will be resumed:

$$\text{(B)}\quad \begin{array}{ll} (4) & \text{Substrate} + \text{methylene blue} \underset{\text{dehydrase}}{\rightleftarrows} \text{oxidized substrate} \\ & \qquad\qquad\qquad\qquad\qquad\qquad + \text{leuco methylene blue} \\ (5) & \text{Leuco methylene blue} + \text{oxygen} \rightleftarrows \text{methylene blue} + H_2O_2 \end{array}$$

The sole difference between this scheme and that given above is that hydrogen peroxide accumulates in B. In other words, oxygen consumption itself is without effect.

Many cells, as mentioned above, do possess a scheme for oxygen utilization unaffected by cyanide. Warburg has recently found evidence for the following train of events:

$$\text{(C)}\quad \begin{array}{ll} (6) & \text{Substrate} + \text{coenzyme} \underset{\text{dehydrase}}{\rightleftarrows} \text{oxidized substrate} \\ & \qquad\qquad\qquad\qquad\qquad\qquad + \text{reduced coenzyme} \\ (7) & \text{Reduced coenzyme} + \text{yellow pigment} \rightleftarrows \text{coenzyme} \\ & \qquad\qquad\qquad\qquad\qquad\qquad + \text{reduced yellow pigment} \\ (8) & \text{Reduced yellow pigment} + \text{oxygen} \rightleftarrows \text{yellow pigment} + H_2O_2 \\ (9) & H_2O_2 \underset{\text{catalase}}{\rightleftarrows} H_2O + 1/2\, O_2 \end{array}$$

In the presence of cyanide, only (9) is inhibited. Therefore, in organisms possessing analogous respiratory mechanisms, cyanide should act similarly to its effect on *E. coli* in the presence of a dye; according to Burnet (1927), this seems to be the case for *Staphylococcus* which in the presence of cyanide, grows under anaerobic conditions while not tolerating aerobiosis.

At first sight, these facts appear cogent arguments in favor of the thesis of M'Leod and Gordon (1923a, b) that the intolerance for oxygen displayed by obligate anaerobes is due to their produc-

tion of H_2O_2 in the presence of this gas. Such, however, can not be the case as was stated at the outset. In the first place, peroxide production can not be chemically detected in cultures of obligate anaerobes exposed to oxygen. Hydrogen peroxide may conceivably arise not only from the reduction of molecular oxygen, but also under anaerobic conditions from either of the following reactions:

$$(10)\ 2H_2O \rightleftarrows H_2O_2 + 2H^+ + 2e$$

$$(11)\ 2OH^- \rightleftarrows H_2O_2 + 2e$$

In both of these schemes, H_2O_2 is the oxidant of a system of high potential and much work would indicate that such high potentials are incompatible with life. In other words, neither water nor the hydroxyl ion could be possibly oxidized to peroxide in the presence of a living cell. On the other hand, oxygen may readily be reduced. Furthermore, in table 7 it is demonstrated that *E. coli*, in the absence of oxygen consumption, is unable to form peroxide. It may, therefore, be assumed that oxygen consumption must precede peroxide formation and there has never been adduced any evidence whatsoever for any mechanisms such as "A" "B" or "C" in obligate anaerobes. To produce the amounts of peroxide found necessary to inhibit growth, amounts of oxygen more than sufficient to be detected manometrically must be consumed. Yet obligate anaerobes have never been found to consume any amount of this gas (Fujita and Kodama (1934); Stickland (1935b)). In the case of such organisms as the pneumococcus and of *E. coli* growing in the presence of cyanide and dye, large amounts of oxygen consumption and peroxide formation may be detected and inhibition may properly be attributed to this factor. In the former organism, the relative tolerance towards H_2O_2 allows growth to proceed until amounts of peroxide sufficient to inhibit have accumulated.

The ready growth that occurs under aerobic conditions when *E. coli* is grown in cyanide media appears to cast grave doubt on the currently assumed rôle of potential in anaerobic growth. If anaerobic proliferation depended on the absence of oxygen solely because the latter raised the potential of a medium (Quastel),

cyanide-treated bacteria should not grow in oxygen. The reactions they catalyze (sugar dissimilation) have a much more negative potential than is the case with some obligatory anaerobes. Actually oxygen is not inhibitory because it cannot enter into the reaction. The following considerations make this obvious.

If a platinum or gold electrode be inserted in a sterile culture medium, and this be used as a half cell in a concentration chain, a relatively high potential will be found under aerobic conditions. If anaerobiosis is induced, either by evacuation or introduction of an inert gas, a lower potential is observed. If the culture is inoculated and growth starts in oxygen, still lower potentials may be recorded, whereas, if anaerobiosis is secured, extremely negative potentials are likely.

One important fact appears to us to have been often neglected. The metallic electrodes used partially catalyze the attainment of equilibrium of the oxygen electrode. There is no perfect catalyst for this half cell and equilibrium is never attained. Nevertheless, in the presence of an electrode, molecular oxygen is enabled to exert some fraction of its theoretical potential (i.e., of its oxidizing power). This is tantamount to a statement that an electrode introduces conditions which had not previously existed—i.e., raises the *potential*. These considerations raise the highly theoretical question as to what potential exists in the absence of an "interpreting" electrode.[6] Actually, reduction potentials are but a form of expression of the free energy changes of a system. Most biological systems, including oxygen, require catalysis of some sort before this free energy is available and, even if such systems are present, they can not influence the free energy level unless their specific catalysts are also present. In other words, little can be concluded from the potential of a sterile medium[7] in the absence or presence of oxygen. The introduction

[6] In this connection, see the interesting discussion by Cohen, Michaelis, and MacInnes in Cohen (1933).

[7] That is, a medium containing no electromotively active oxidation-reduction systems and devoid of metallic catalysts. In practice, such an ideal system is difficult to realize.

of an electrode in oxygen enables a potential to be read that can be interpreted only with regard to the extremely imperfect attainment of equilibrium of the oxygen electrode.

In the case of an obligate anaerobe it is appreciated that certain reactions are catalyzed which may or may not lead to a reproducible potential depending on whether or not the systems actually attain a steady state. Let us assume such has been attained and a given potential is recorded in the absence of oxygen, by an electrode. Now, if oxygen is introduced, a much higher potential may be read depending on the relative velocities of the reaction under consideration and of the attainment of equilibrium of the oxygen system at the metallic electrode. Suppose, however, no electrode is present when oxygen is admitted. Provided there are present no catalysts for the "activation" of oxygen, and no substances of this sort (schemes A, B or C) have been found present in strict anaerobes, the effect should be the same as if oxygen were not present; i.e., the latter gas is chemically "inert" in regard to the reaction under consideration. In other words, the true potential of the cellular environment is not disturbed by the admission of oxygen, but is apparently affected by the introduction of an electrode.

Such considerations suggest that the latent oxidizing power of oxygen can not explain its inhibition of growth by obligate anaerobes unless it actually oxidizes the cellular protoplasm and as yet, there is little evidence for such an assumption. An alternative explanation is that oxygen forms some loose chemical complex with the anaerobic system of obligate anaerobes and thus removes it from activity. This phase of the problem is now under investigation.

Our data may have some bearing on such problems as the nature of the inhibition of fermentation by dye systems as discussed by Lipmann (1934) and Michaelis and Smythe (1935). In this connection we have obtained evidence that hydrogen peroxide may decrease the activity of certain enzymic systems and will consider this with some other aspects in a subsequent paper.

SUMMARY AND CONCLUSIONS

1. The lethal action of cyanides on certain bacteria reported as being due to the accumulation of peroxide is attributed to other causes.

2. Cyanides permit anoxytrophic growth of *Escherichia coli* to proceed but prevent oxytrophic multiplication.

3. Reversible oxidation-reduction systems of suitable potential completely arrest growth in the presence of oxygen and of cyanide but have no marked action under anaerobic conditions. This effect is traced to the accumulation of peroxide under aerobic conditions.

4. The anaerobic system of facultative anaerobes is shown to be fundamentally distinct from that of obligate anaerobes.

5. The mechanism of obligate anaerobiosis has been discussed and it is demonstrated that neither the peroxide theory of M'Leod and Gordon nor the reduction potential theory of Quastel and others is tenable.

REFERENCES

BARRON, E. S. G., AND HAMBURGER, M. 1932 The effect of cyanide upon the catalytic action of dyes on cellular oxygen consumption. Jour. Biol. Chem., 96, 299.

BARRON, E. S. G., AND HOFFMAN, L. A. 1930 The catalytic effect of dyes on the oxygen consumption of living cells. Jour. Gen. Physiol., 13, 483.

BUCHANAN, J. W. 1926 Depression of oxidative metabolism and recovery from dilute potassium cyanide. Jour. Exp. Zool., 44, 285.

BURNET, F. M. 1927 The action of cyanides on bacteria. Jour. Path. Bact., 30, 21.

CLARK, W. M. 1934 The potential energies of oxidation-reduction systems and their biochemical significance. Medicine, 13, 207.

COHEN, B. 1933 Reversible oxidation-reduction potentials in dye systems. Cold Spring Harbor Symposium Quant. Biol., 1, 195.

DACK, G. M., AND BURROWS, WM. 1935 Oxidation-reduction potentials of some nonsporulating obligate anaerobes. Proc. Soc. Exp. Biol. and Med., 32, 1441.

FLEISCH, A. 1924 Some oxidation processes of normal and cancer tissue. Biochem. Jour., 18, 294.

FUJITA, A., AND KODAMA, T. 1934 Untersuchungen über Atmung und Gärung pathogener Bakterien. III. Über Cytochrom und das sauerstoff-übertragende Ferment, sowie die Atmungshemmung der pathogenen Bakterien durch CO und HCN. Biochem. Z., 273, 186.

Kluyver, A. J., and Hoogerheide, J. C. 1936 Beziehungen zwischen den Stoffwechselvorgängen von Hefen und Milchsäurebakterien und dem Redox-Potential in Medium. Enzymologia, 1, 1.

Lipmann, F. 1934 Ueber die Hemmung der Mazerationssaftgärung durch Sauerstoff in Gegenwart positiver Oxydoreduktionssysteme. Biochem. Z., 268, 205.

M'Leod, J. W., and Gordon, J. 1923a. Catalase production and sensitiveness to hydrogen peroxide amongst bacteria: With a scheme based on these properties. Jour. Path. Bact., 26, 326.

M'Leod, J. W., and Gordon, J. 1923b. The problem of intolerance of oxygen by anaerobic bacteria. Jour. Path. Bact., 26, 332.

Michaelis, L., and Smythe, C. V. 1935 Influence of certain dye stuffs on fermentation and respiration of yeast extract. Proc. Soc. Exp. Biol. and Med., 33, 127.

Novy, F. G., Jr. 1925 Microbic respiration. IV. The so-called aerobic growth of anaerobes; potato respiration. Jour. Inf. Dis., 36, 343.

Pasteur, L. 1861 Animalcules infusoires vivant sans gaz oxygene libre et determinant des fermentations. Compt. rend. acad. sci., 52, 344.

Quastel, J. H., and Stephenson, M. 1926 Experiments on "strict" anaerobes. I. The relation of B. sporogenes to oxygen. Biochem. Jour., 20, 1125.

Quastel, J. H., Stephenson, M., and Whetham, M. 1925 Some reactions of resting bacteria in relation to anaerobic growth. Biochem. Jour., 19, 304.

Quastel, J. H., and Wheatley, A. H. M. 1931 The action of dyestuffs on enzymes. I. Dyestuffs and oxidations. Biochem. Jour., 25, 629.

Stickland, L. H. 1934 Studies in the metabolism of the strict anaerobes (genus clostridium). I. The chemical reactions by which Cl. sporogenes obtains its energy. Biochem. Jour., 28, 1746.

Stickland, L. H. 1935a Studies in the metabolism of the strict anaerobes (genus clostridium). II. The reduction of proline by Cl. sporogenes. Biochem. Jour., 29, 288.

Stickland, L. H. 1935b Studies in the metabolism of the strict anaerobes (genus clostridium). III. The oxidation of alanine by Cl. sporogenes. Biochem. Jour., 29, 889.

Stickland, L. H. 1935c Studies in the metabolism of the strict anaerobes (genus clostridium). IV. The reduction of glycine by Cl. sporogenes. Biochem. Jour., 29, 896.

Todd, E. W. 1930 Virulence of hemolytic streptococci. I. The influence of oxygen on the production of glossy variants. Brit. Jour. Exp. Path., 11, 368.

DECOMPOSITION OF NITROGENOUS SUBSTANCES IN SEA WATER BY BACTERIA[1]

SELMAN A. WAKSMAN, MARGARET HOTCHKISS, CORNELIA L. CAREY AND Y. HARDMAN

Woods Hole Oceanographic Institution and New Jersey Agricultural Experiment Station

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It has been definitely established that organic nitrogenous bodies are rapidly decomposed in sea water by bacteria and that the nitrogen is liberated as ammonia. Waksman and Renn (1936) studied the oxidation of a series of amino acids in sea water using oxygen consumption as a measure of their destruction and found that, after making allowance for the basic sea water oxidation, the oxygen consumed in 5 days ranged from 50 per cent of the theoretical requirement for complete oxidation in the case of asparagine to 75 per cent in the case of glutamic acid. Measurements made at the same time showed that the increase in bacterial numbers ran parallel to the extent of the oxidation of the various amino compounds. In another experiment (Waksman, Carey, and Reuszer, 1933), it was calculated that in 19 days 1 gram of air-dry zooplankton liberated 100.7 mgm. carbon as CO_2 and 38.5 mgm. nitrogen as NH_3; the C–N ratio in the plankton was 4.57, whereas the corresponding ratio of the two metabolic products was 2.62. These results pointed to a very rapid liberation of the nitrogen as ammonia, in the decomposition of nitrogen-rich plankton, such as copepod plankton, by bacteria. In a third experiment it was found (Waksman and Carey, 1935) that the addition of 50 copepods (*Calanus finmarchicus*) to 225 cc. sea water resulted in an increase in bac-

[1] Contribution No. 160 of the Woods Hole Oceanographic Institution and Journal Series Paper of the New Jersey Agricultural Experiment Station, Department of Soil Microbiology.

terial numbers, in 24 hours, from 229,500 per cubic centimeter in the control water to 17,450,000 per 1 cc.; this was accompanied by an increase of 3.77 cc. in the consumption of oxygen per liter of water in 48 hours. These amounts were comparable to the decomposition figures for 4 mgm. asparagine in an equal amount of sea water.

The studies reported here examine in more detail the relationship between bacterial multiplication, oxygen consumption and nitrogen liberation, in the decomposition of nitrogenous material in sea water. Copepods were again used as representative of the nitrogen-rich marine zooplankton and asparagine as a type of nitrogenous organic compound.

The oxygen was determined by the Winkler method, the nitrate by the reduced strychnine method, the phosphate by the Atkins-Denigés method, and the bacteria by plate and microscopic methods. The ammonia was determined in the first experiment by the method of Krogh and in the other two experiments by Nessler's method.

In the first experiment (table 1) different numbers of fresh *Calanus finmarchicus* were added to a series of oxygen bottles containing freshly-taken sea water. The bottles were incubated, under water, in the dark and at room temperature, for a period of five days. Control bottles gave the decomposition figures for the basic metabolism of the sea water and of the sea water plus glucose, since the oxidation of glucose has been found to be a useful measure of the readily available nitrogen in sea water (Waksman and Carey, 1935). Other control bottles showed the decomposition figures for the sea water plus a nitrate source of nitrogen, with and without the addition of glucose as an extra source of carbon. According to this experiment, *Calanus finmarchicus* contains material readily decomposable by bacteria: a fact which was measured directly by determining the liberation of ammonia and phosphate and the consumption of oxygen, and indirectly by noting the increased oxygen consumption in those bottles which contained glucose.

From results presented in table 1, it is apparent that different amounts of copepod material apparently liberate nitrogen and

TABLE 1

Decomposition of Calanus finmarchicus by bacteria

NATURE OF MATERIAL			ANALYTICAL DATA					
Calanus per bottle*	Glucose	Nitrate nitrogen	Oxygen consumed		Bacteria in 1 cc.	Ammonia-N	Phosphate	Nitrate-N
			2 days	5 days	2 days	5 days	5 days	2 days
	mgm. per liter	*mgm. per liter*	*cc. per liter*	*cc. per liter*	*thousands*	*mgm. per liter*	*mgm. per liter*	*mgm. per liter*
0	0.0	0.0	0.36	0.54	151	0.02	0.045	0.01‡
0	0.0	0.07	0.30		180			0.08
2	0.0	0.0	0.66	1.08	285	0.08	0.06	
5	0.0	0.0	1.08	2.16	930	0.18	0.10	
15	0.0	0.0	2.01	3.78	1,950	0.31	0.15	
0	5.0	0.0	0.69	1.32	125			0.01
0	5.0	0.07	2.34	3.30	605			0.01‡
2	5.0	0.0	2.43	2.82	1,100			
5	5.0	0.0	2.49	3.64		0.09		
15	5.0	0.0	4.32	4.98†	950	0.22		

* 4.5 bottles to the liter.
† Oxygen completely used up.
‡ The same amounts found after 5 days.

phosphorus in the same ratio as the parallel figures show: for 2 copepods per bottle the ratio was,

$$\frac{N}{PO_4} = \frac{0.08 - 0.02}{0.06 - 0.045} = 4.0:1$$

for 5 copepods,

$$\frac{N}{PO_4} = \frac{0.18 - 0.02}{0.10 - 0.045} = 3.0:1$$

for 15 copepods,

$$\frac{N}{PO_4} = \frac{0.31 - 0.02}{0.15 - 0.045} = 2.8:1.$$

Orr (1933–34) has shown that it takes 3.5 to 10 grown *Calanus finmarchicus* to give 1 mgm. of dry material, and that the protein content of these copepods varies from 40 to 70 per cent for the males and for the females might be as much as 75 per cent. Assuming that a single copepod weighs 0.1 to 0.2 mgm. and that

its cell substance contains 10 per cent nitrogen, one animal would contain 0.01 to 0.02 mgm. of nitrogen; nine copepods per liter of water,[2] on this assumption, would have contained 0.09 to 0.18 mgm. of nitrogen. The amounts of ammonia liberated in the above experiment are in accord with this theoretical nitrogen calculation; namely, 0.06 mgm. nitrogen was liberated as ammonia for an equivalent of nine copepods per liter (2 per bottle), 0.16 mgm. for two and a half times this number, and 0.29 mgm. for the largest quantity of copepod material, after subtracting the control. The corresponding amounts of oxygen consumed were, after allowing for the water control, 0.54, 1.62 and 3.24. In other words, both ammonia liberation and oxygen consumption were parallel to the number of copepods added to the bottles.

When glucose was added to the bottles, there was an increase in oxygen consumption of 1.50 cc. in the presence of 2 copepods, 2.32 cc. in the case of 5 copepods, and a larger amount with the highest number of copepods. When, in place of copepods, nitrate was added to the water containing glucose, at the rate of 0.07 mgm. nitrogen per liter, the increase in oxygen consumption was about the same as with 5 copepods. In other words, the bacteria consumed, for the oxidation of 5 mgm. glucose, 0.07 mgm. nitrogen as nitrate, which was transformed into bacterial protein. It is of special interest to note that when 5 mgm. of glucose were added to the water containing 5 copepods, the reduction in the amount of nitrogen liberated as ammonia was 0.09 mgm. per liter.

In another experiment it was found that the consumption of phosphorus by bacteria, using 5 mgm. of glucose, was equivalent to 0.06 mgm. phosphate. In other words, in the decomposition of glucose, the bacteria consumed 0.06 mgm. phosphate for the synthesis of their cell substance. The fact that the consumption of nitrogen, in the decomposition of 5 mgm. glucose, was 0.09 mgm. indicates that the ratio between nitrogen and phosphate requirements for bacterial metabolism in the sea is 1.5:1. Since the bacteria multiplied most rapidly under the conditions where

[2] Since each bottle contains 220 to 225 cc. water, 2 copepods per bottle will be equivalent to 9 per liter of water.

oxygen consumption was greatest, and since the rate of multiplication and oxygen consumption was high where copepods were added, it seems beyond question that the bacteria can play an important part in the decomposition of animal plankton under natural conditions.

In two later experiments the decomposition of asparagine was studied in greater detail. In one of these experiments (table 2), oxygen consumption and bacterial multiplication in fresh sea water were compared with oxygen consumption and bacterial multiplication in the same water plus 5 mgm. asparagine per liter. A series of oxygen bottles was filled with each type of water; they were incubated in the dark and at room temperature

TABLE 2

Decomposition of asparagine by bacteria in fresh sea water

PERIOD OF INCUBATION	SEA WATER			SEA WATER + ASPARAGINE, 5 MGM. PER LITER			
	Oxygen consumed	Bacteria in 1 cc.	Microscopic counts per field	Oxygen consumed	Bacteria in 1 cc.	Microscopic counts per field	$NH_3 - N$
days	*cc. per liter*	*thousands*		*cc. per liter*	*thousands*		*mgm. per liter*
1	0.46	656.0	54	1.31	3,340.0	125	
2	0.75	457.0	52	2.13	2,465.0	100	
4	1.19	16.0	30	2.84	53.5	49	
6	1.33	31.0		3.00	30.5		0.70
10	1.28	5.4		3.43	9.0		0.60

under water, and, at frequent intervals, several bottles were removed for the determination of bacteria by the plate method and for chemical analysis. In both the control and the asparagine-enriched water, the greatest bacterial population was found in the one and two day incubation periods, and the rate of oxygen consumption was highest during the first four days of incubation. The asparagine-enriched water, at its peak, contained approximately five times as many bacteria per cubic centimeter and consumed, in four days, over twice as much oxygen. The high degree of oxygen consumption and bacterial multiplication indicate that the decomposition of practically all the asparagine used occurred in four days. The ammonia liberated was 60 to 70 per

cent of the total nitrogen added in the form of asparagine. The excess oxygen consumption was 2.15 cc. in ten days. The theoretical amount of oxygen required to oxidize 5 mgm. of asparagine, with the liberation of nitrogen as ammonia, is 2.50 cc.; hence, only 86 per cent of the theoretical amount of oxygen was utilized in the oxidation of the asparagine; the rest of it presumably being consumed by the bacteria in 10 days for the synthesis of their cell substance.

TABLE 3

Decomposition of different amounts of asparagine in cultured sea water by bacteria

Incubation	Control sea water			Sea water + 5 mgm. asparagine per liter			Sea water + 10 mgm. asparagine per liter			Sea water + 20 mgm asparagine per liter		
	Oxygen consumed	$NH_3 - N$	Bacteria in 1 cc.	Oxygen consumed	$NH_3 - N$	Bacteria in 1 cc.	Oxygen consumed	$NH_3 - N$	Bacteria in 1 cc.	Oxygen consumed	$NH_3 - N$	Bacteria in 1 cc.
days	*cc. per liter*	*mgm. per liter*	*thousands*	*cc. per liter*	*mgm. per liter*	*thousands*	*cc. per liter*	*mgm. per liter*	*thousands*	*cc. per liter*	*mgm. per liter*	*thousands*
1	0.10	0.01	54.0	0.78	0.50	520.0	0.42	0.13	410.0	0.33	0.06	435.0
2	0.16	0.01	345.0	1.58	0.65	2,515.0	2.97	0.85	2,900.0	4.55*	1.10	965.0†
3				2.09			3.36	1.20		5.36	1.70	
4	0.33	0.01	63.0	2.34	0.72	40.5	3.60	1.40	234.0	6.59	2.25	683.0
6	0.59	0.04	27.3	2.34	1.00	13.4	3.89	1.40	33.7	7.37	2.75	60.5
9				2.58	0.90	6.6	4.19		9.4	8.80	3.50	23.0

* Water transferred from oxygen bottles to clean, glass-stoppered bottle, shaken to resaturate with oxygen and returned to original bottles.

† Low oxygen tension unfavorable to aerobic bacteria.

Considerable oxygen is consumed by the bacteria in fresh sea water acting upon its organic matter, hence the water was allowed to reach a certain stability by holding it in the laboratory. A quantity of the fresh sea water was filtered through a No. 20 silk net and allowed to remain in an 18-liter glass bottle in the laboratory for 9 days. The water was then thoroughly aerated, in order to resaturate it with oxygen. This "cultured" water was divided into four lots, one of which was left unchanged, while to the other three lots were added respectively 5, 10 and 20 mgm. portions of asparagine per liter. The four lots were distributed in 225 cc. oxygen bottles and incubated under water,

in the dark. Analyses were made at definite intervals, using duplicate or triplicate bottles.

The results, presented in table 3, point to the fact that asparagine is completely decomposed in sea water in 6 to 9 days, since in that time all the nitrogen in the asparagine became liberated as ammonia. There was also a rapid drop in bacterial numbers. Microscopic examination (table 4) revealed the fact that, after 9 days, most of the bacteria had disappeared leaving a mass of débris in which various protozoa abounded. Oxygen consumption slowed down considerably after 6 days, especially with the lower concentration of asparagine.

TABLE 4

Microscopic population of bacteria in sea water enriched with asparagine (table 3)

Number of cells per field

INCUBATION	CONTROL WATER	5 MGM. ASPARAGINE PER LITER	10 MGM. ASPARAGINE PER LITER	20 MGM. ASPARAGINE PER LITER
days				
1	12	34	39	69
2	48	124	96	79
3		28	43	44
4	10*	26	102	153
6	9	41	40	82
9		4	19	Débris

* One slide only, the other had very few bacteria.

The 20-mgm. amount of asparagine was at first oxidized slowly, but later was rapidly attacked by the bacteria so that, within 48 hours, the oxygen content in the water was reduced to a minimum. This produced an abnormal state for bacterial development, as could be demonstrated by the fact that the microscopic examination of the slides revealed the bacterial cells in a much swollen condition, resembling involution forms. The water in the remaining bottles containing the highest amount of asparagine was carefully transferred to clean glass-stoppered bottles; these were well shaken in order to resaturate the water with oxygen; the water was then transferred back to the original oxygen bottles, 3 to 4 drops of cultured water being used to close the glass seal. For a six-day period, 5 mgm. of asparagine in-

creased the consumption of oxygen 1.75 cc. per liter; 10 mgm. of asparagine increased the consumption 3.30 cc. per liter, and 20 mgm. of asparagine 6.78 cc. per liter. The consumption of O_2 thus increased in exact proportion with the increase in asparagine. These results point to the fact that about 70 per cent of the theoretical requirement of oxygen for the complete oxidation of the asparagine was consumed by the bacteria in 6 days. The residual carbon was probably stored in the bacterial cell substance, in a manner similar to bacterial synthesis with glucose as a source of energy. The later oxygen consumption took place at the expense of these synthesized bacterial cells, which were rapidly disintegrated either by processes of autolysis or through consumption by protozoa.

In order to demonstrate microscopically the changes in the bacterial population in sea water and in water enriched with asparagine in the above experiments, two clean glass slides were placed in the oxygen bottles and examined microscopically, whenever the bottles were removed for analysis. The slides were air-dried, fixed 3 to 4 times in a flame, and stained for one minute in Hucker's crystal violet. The counts were made with an oil immersion lens. The fields measured 0.0082 square millimeter. All the bacteria were counted in five fields per slide and the averages for 10 fields (or 2 slides) reported.

The results obtained in the first experiment (table 2) show that plate and microscopic methods for the determination of bacteria present a similar picture; in both cases marked reductions in numbers were observed at 4 days incubation. While the plate method showed at that time a sudden dying off of the bacteria, the microscopic method, which does not distinguish between living and dead cells, did not show as rapid a reduction. However, after 6 days, the slides were covered with débris and the bacteria could no longer be counted.

Similar results were obtained in the second experiment (table 4). After a rapid rise, the numbers of bacteria rapidly decreased, so that after 9 days incubation, the cells could no longer be distinguished.

These results confirm those previously presented (Waksman

and Hotchkiss, 1936) in showing that after a certain period the bacterial population in sea water will rapidly drop off in numbers. No matter how high the numbers become and no matter how the increase is brought about, it is soon followed by a rapid drop, due to autolytic processes and activities of the animal population.

SUMMARY

1. Copepods were rapidly decomposed in sea water by bacteria; this was accompanied by active bacterial multiplication, oxygen consumption, liberation of nitrogen as ammonia and phosphate regeneration.

2. Nitrogen liberation by bacteria in sea water, as measured by increased consumption of oxygen as a result of addition of glucose, gave equivalent values when measured directly as ammonia.

3. Seventy per cent of the theoretical amount of oxygen required to oxidize asparagine completely was consumed by the bacteria in 6 days, during the decomposition of the asparagine in sea water, and over 80 per cent in 10 days.

4. The bacterial maximum in incubated sea water or in water receiving asparagine was attained in 2 days, as determined by the plate method, and in 2 to 4 days, as determined by the microscope. This corresponded to the time of the maximum rate of asparagine decomposition.

5. The rise in bacterial numbers was followed by a sudden drop, after the nutrients added to the water had been exhausted. The drop was demonstrated by the plate method at an earlier incubation period than by the microscope.

The authors are indebted to Dr. C. E. Renn for making the ammonia determinations reported in table 1.

REFERENCES

Orr, A. P. 1933-1934 The biology of *Calanus finmarchicus*. IV. Seasonal changes in the weight and chemical composition in Loch Fyne. Jour. Mar. Biol. Assoc., 19, 613-632.

Waksman, S. A., and Carey, C. L. 1935 Decomposition of organic matter in sea water by bacteria. II. Influence of addition of organic substances upon bacterial activities. Jour. Bact., 29, 545-561.

WAKSMAN, S. A., CAREY, C. L., AND REUSZER, H. W. 1933 Marine bacteria and their rôle in the cycle of life in the sea. Biol. Bull., 65, 57-79.

WAKSMAN, S. A., AND HOTCHKISS, M. 1937 Viability of bacteria in sea water. Jour. Bact., 33, 389-400.

WAKSMAN, S. A., AND RENN, C. E. 1936 Decomposition of organic matter in sea water by bacteria. III. Factors influencing the rate of decomposition. Biol. Bull., 70, 472-483.

GROWTH RATES OF PHYTOPATHOGENIC BACTERIA

E. M. HILDEBRAND[1]

Department of Plant Pathology, New York State College of Agriculture, Ithaca, New York

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In connection with another investigation concerned with strain variations (Hildebrand ms.) some growth rate studies were made of several isolates of the fire-blight organism, *Erwinia amylovora*. For purposes of comparison similar studies were also made of a number of *Phytomonas* species and, because of the paucity of such data in phytopathological literature, it was thought worthwhile to report briefly the results.

In these studies the period of lag, or the latent period through which a freshly inoculated bacterial culture passes before entering its period of rapid growth, was largely eliminated by making from one to three transfers of the test bacteria at daily intervals before starting the experiment.

For the actual measurement, 1 cc. of a 1:10,000 dilution of these young cultures was transferred to 100 cc. of nutrient broth (pH = 6.6). Nutrient agar (pH = 6.6) was largely used for the plate counts. In a few instances, indicated later, special media in liquid and solid form were employed (nutrient-glucose, potato-glucose, and potato-mannitol containing 0.5 per cent glucose or mannitol).

Tests were made of 14 different isolates of the fire-blight organism. The culture numbers are abbreviations of the states or countries serving as sources. For example, the symbol NZ7 represents the seventh of a series of cultures obtained from New Zealand and NZ7d represents a culture which is the progeny of the fourth or "d" single cell isolated from the above culture.

[1] The writer is indebted to the Department of Bacteriology and Dairy Industry for kindly advice and the use of their facilities and to Messrs. P. A. Smith and R. F. Brooks for assistance in making these studies.

Tests were also made of 11 Phytomonas species including *P. apii*, *P. fascians*, *P. flaccumfaciens*, *P. michiganense*, *P. phaseoli*, *P. pruni*, *P. rhizogenes*, *P. syringae*, *P. tumefaciens*, *P. utiformica*, and an unnamed species causing cane gall of black raspberry.

Plate counts were made at three-hour intervals for 15 hours, after which the usual intervals were 24, 30, 36, 48, 60 and 72 hours. These platings covered the first three of the four phases of growth. The logarithmic growth phase is indicated in table 1 for the different bacterial species employed in this investigation. The plates were counted after 48 hours.

The generation times were determined graphically, using the "growth-rate protractor" of Rahn and Mason (1935).

From the data given in table 1 it is seen that generation times ranged from 55 to 155 minutes for the 25 cultures studied. Considerable variation may be observed within a single species since for *Erwinia amylovora*, involving 14 different isolates, the range was from 71 to 94 minutes, with an average of 82.0 minutes. The maximal deviations are -13.4 per cent and $+14.6$ per cent.[2] The greatest difference, of 23 minutes between strains of widely different origin, is not much greater than that of 17 minutes between "parent" Ont. 3 and "progeny" Ont. 3b. with respectively 71 and 88 minutes. The average deviation between parent and progeny isolates of the several strains was -1 ± 2.8 minutes.

Despite the preliminary transfers, culture N. Y. 8 was not out of its lag phase because 12 hours were required before its logarithmic phase was reached.

Of the 11 *Phytomonas* species studied, only one, *P. utiformica*, had a generation time under one hour (55 to 59 minutes) and *P. syringae* which is considered by some to be the same species as the above comes next with 73 minutes. *P. apii*, *P. flaccumfaciens*, and *P. tumefaciens* have practically identical generation times ranging from 78 to 85 minutes which represents roughly the mean for *Erwinia amylovora*. Four species—*P. phaseoli*, *P. pruni*, *P. michiganense*, and *P. rhizogenes*—have generation

[2] For the 15 minute generation time of *Bacterium coli* this percentage would correspond to a fluctuation of ± 2 minutes.

TABLE 1

Maximum growth rates of some plant-pathogenic species of bacteria including several strains of Erwinia amylovora and members of the genus Phytomonas at 30°C.

SPECIES	CULTURE NUMBER	GENERATION TIME	CULTURE NUMBER	GENERATION TIME	DEVIATION*	PERIOD	MEDIA (BROTH)
		minutes		*minutes*	*minutes*	*hours*	
E. amylovora	Cal 1	86	Cal 1c	79	−7	3–30	Nutrient
	NY 3	79	NY 3a	83	+4	6–24	Nutrient
	NY 8	94	NY 8b	82	−12	6–24‡	Nutrient
	NY 10		NY 10a	94		3–15	Nutrient
	NZ 7	78	NZ 7d	74	−4	3–18	Nutrient
	Ont 3	71	Ont 3b	88	+17	3–15	Nutrient
	Va 3	78	Va 3a	77	−1	3–15	Nutrient
			Va 3a	72†	−6	3–15	Nutrient
			Va 3c	80	+2	3–15	Nutrient
			Va 3c	82†	+4	3–15	Nutrient
			Va 3a	90		3–15	Nutrient-glucose
P. utiformica	B	55				6–15	Nutrient
P. utiformica	H	59				6–15	Nutrient
P. syringae		73				6–15	Nutrient
P. tumefaciens		78				6–15	Nutrient
P. apii		80				10–24	Nutrient
P. flaccumfaciens		80				12–24	Nutrient
P. fascians		88				12–24	Nutrient
P. michiganense		115				12–36	Nutrient
P. rhizogenes		121				12–36	Nutrient
P. pruni		126				10–24	Nutrient
P. phaseoli		134				10–24	Nutrient
Cane gall		155				10–24	Nutrient
P. utiformica	H	78				6–15	Nutrient-glucose
P. tumefaciens		85				6–15	Potato-mannitol
P. rhizogenes		112				12–36	Potato-mannitol
P. pruni		122				10–24	Potato-glucose

* The average deviation was found to be −1 ±2.8 minutes.

† Generation time one year later.

‡ The logarithmic growth period of NY 8 was 12 to 36 hours which is considerably slower than NY 8a or the other *E. amylovora* isolates.

times in the neighborhood of two hours. The cane-gall organism has the slowest rate of reproduction, of slightly over two and one-half hours.

The effect of adding sugar to the media was demonstrated for two species, *Erwinia amylovora* (Va. 3a) and *P. utiformica.* In both instances the generation times were increased, respectively, from 77 to 90 and from 59 to 78 minutes. These results are not in agreement with those of Mason (1935) who obtained an opposite effect. With cultures grown in broth and glucose broth, he obtained generation times of 150 and 138 minutes for *P. phaseoli*, 98 and 78 minutes for *P. campestris* and 57 and 42 minutes for *Erwinia carotovora.* The reasons for these differences are not readily apparent although it is possible that certain species will react differently than others to additions of sugar to media.

From the standpoint of special media potato-glucose (composed of potato extract and the sugar) proved more favorable than nutrient medium for *P. pruni*, and potato-mannitol proved more favorable than nutrient medium for *P. rhizogenes*, the decreases in generation times being respectively 4 and 9 minutes, but in the case of *P. tumefaciens* potato-mannitol had the opposite effect in causing the generation time to increase by 7 minutes. Based on the experiments with *Erwinia amylovora* these differences may be within experimental error.

The loss of pathogenicity in *E. amylovora* seemed to have an effect on generation time. The generation times of Va 3a and Va 3c were measured both in 1936 and 1937. In 1936 the Va 3a culture in contrast to the highly pathogenic Va 3c culture had almost lost its virulence, being incapable of producing more than a slight necrosis in green pear fruits. By 1937 it had become completely non-pathogenic whereas the Va 3c culture remained about the same as before. During this period of one year the generation time of the Va 3a culture was shortened and that of the Va 3c culture slightly lengthened. While the significance of this observation is not certain in this instance, loss of pathogenicity may be correlated with a decrease in generation time.

DISCUSSION

Since the chief purpose of this study was to determine relative rates of growth rather than exact maxima, no special claims are made for these data as representing maximum possible rates of growth. Moreover, the incubation temperature was arbitrarily set at 30°C. which is above the optima found in literature for some of these species. The precise determination of the optimal temperatures for the growth rates remains for future work. However, 30° is near the optimal temperature, and a change of a few degrees will not make much difference in the growth rate at this point. Doubtless, special media might have promoted faster growth and evidence for this is given in table 1.

The outstanding result of this investigation is that the plant-pathogenic species of the two main groups studied are unusually slow-growing bacteria. Mason listed 51 species of which 12 (or 23 per cent) required more than 45 minutes for a cell division. Of these, 7 (or 14 per cent) were plant pathogens. To this list, 1 *Erwinia* and 11 *Phytomonas* are added in this paper all of which are slow-growing. This gives a total of 2 *Erwinia* and 18 *Phytomonas* species all of which have a remarkably slow reproduction rate, from 0.75 to 2.5 hours, while of the non-plant-pathogenic bacteria listed by Mason, 88 per cent can divide in less than 45 minutes.

The genus *Phytomonas* is doubtless very closely related to *Pseudomonas* which has a generation time of about 30 minutes, being slower than the average of all bacteria. The relation of *Erwinia* is less certain.

SUMMARY

The growth rate of 7 strains of *Erwinia amylovora* has been determined in nutrient broth. The generation time varied between 71 and 94 minutes, with an average of 82 minutes. The deviation between the original strain and single-cell isolates amounted to 17 minutes in the extreme. The average deviation between parent and progeny isolates was -1 ± 2.8 minutes.[3]

[3] That is, one-half of such comparisons will differ less than 6.2 minutes, and one-half more than 6.2 minutes.

The species is definitely more slow-growing than *Erwinia carotovora*.

Eleven species of *Phytomonas* grown in nutrient broth showed generation times ranging from 55 to 155 minutes. Considering the computation of variability for *Erwinia amylovora* it seems that at least two groups can be distinguished, one requiring between 1.0 and 1.5 hours, and the other between 2 and 2.5 hours.

While the use of glucose and of special media apparently had some effect on generation time of certain species it is possible that the observed differences are not significant and may be open to another interpretation.

REFERENCES

HILDEBRAND, E. M. Strains of the fire-blight organism. Ms.

MASON, M. M. 1935 A comparison of the maximal growth rates of various bacteria under optimal conditions. Jour Bact., 29, 103-110.

RAHN, O., AND MASON, M. M. 1935 A protractor for computing the growth rate of bacteria. Jour. Bact., 29, 99-102.

A METHOD FOR THE CONCENTRATION OF POLIOMYELITIS VIRUS IN NASO-PHARYNGEAL WASHINGS[1]

JOHN R. PAUL

WITH THE TECHNICAL ASSISTANCE OF GEORGE BLEZINGER

From the Desert Sanatorium of Southern Arizona, Tucson, and the Department of Medicine, Yale University School of Medicine, New Haven, Connecticut

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Isolation of the virus of poliomyelitis from the nasopharynx of patients with this disease is not readily accomplished. Most attempts result in failure although it is still a question whether the frequency of these failures is due to the rarity of the virus in this site, or to unsatisfactory technique. Consequently, in an effort to improve the technique of testing nasopharyngeal washings for this purpose, these experiments have been undertaken.

EXPERIMENTAL

The experiments consist in the addition of poliomyelitis virus to normal nasopharyngeal washings and the subsequent titration of the minimal infective dose of virus in this medium in unconcentrated samples, and in samples concentrated by freezing and drying through the agency of the Flosdorf-Mudd apparatus (1935). This particular method was chosen because it seemed to be the most adequate for dealing with virus in fluid containing bacteria, where it is important that bacterial growth be inhibited during the concentrating process. Furthermore, the principle involved has a successful precedent in the experiments of Kramer and his associates (1936) whose work indicates that poliomyelitis virus which has been treated with ether, frozen and partially desiccated, will survive this process, although it was

[1] The expenses of this study have been defrayed by a grant from the President's Birthday Ball Commission for Infantile Paralysis Research.

not evident from their work whether the virus was actually concentrated or not.

Other methods of concentrating the virus of poliomyelitis, of which a number have been described, might have been chosen but nearly all of them have been used in media other than nasopharyngeal washings. We have few data as to whether or not they would be effective for the purpose indicated in this study.

Selection of virus. A strain of fairly low intracerebral virulence was purposely chosen for this work. Known as the Wfd. strain, it had been isolated from a fatal case in the California epidemic of 1934. In the present experiments it was used in its 13th, 14th, and 15th monkey passages where its intracerebral action was fairly similar to that observed in earlier passages, which has been described (Trask, Paul *et al.*, 1937). An intracerebral dose of 0.5 cc. of a 1 per cent suspension usually produced experimental poliomyelitis after an incubation period of five to eight days. The disease was definite, though seldom fatal to the monkey.

In preparing the virus suspensions, glycerinized samples of brain and cord from one to three monkeys were used. Small pieces from the midbrain, medulla and various levels of the spinal cord were always included. This material was ground with sterile sand in sterile distilled water to make a 20 per cent suspension. This was centrifuged for 3 minutes at low speed and equal volumes of the supernatant fluid and nasopharyngeal washings were mixed to make a 10 per cent suspension.

Nasopharyngeal washings. These were obtained from two normal children (aged 4 and 5 years) using 100 cc. of sterile distilled water as the irrigating medium. The child was made to gargle a portion of this water and some of it was also washed through the nasopharynx from a syringe inserted into one of the nares. The usual amount of washings collected for each experiment was about 75 cc. The material thus obtained was generally quite turbid from the presence of mucopurulent material.

Removal of bacteria. Before inoculating this material into the brain of a monkey it is necessary to remove (or reduce the number of) bacteria, if a bacterial infection is to be avoided. In previous

clinical work we have used the addition of phenol (Paul and Trask, 1932; Paul, Trask and Webster, 1935) or ether (Paul and Trask, 1932) and we have also filtered the washings (Paul, Trask and Webster, 1935) for this purpose. At first, phenol and ether were both employed in these experiments but the latter eventually proved to be the bactericidal agent of choice. One of its main advantages is that owing to its volatility, ether can be readily removed from the washings after its bactericidal action has taken effect.

An estimate of the effectiveness of ether as a bactericidal agent when added to nasopharyngeal washings can be made from

TABLE 1

Bactericidal effect of ether upon nasopharyngeal washings

	CONCENTRATION OF ETHER				
	2 per cent	5 per cent	8 per cent	10 per cent	12 per cent
Before addition of ether	++++	++++	++++	++++	++++
After exposure to ether for:					
10 minutes	++++	++	+++	+	0
30 minutes	+++	++	+++	+	0
1 hour	++	++	++	0	0
4 hours	+	+	+	0	0

++++ indicates heavy growth of one loopful, plated on blood agar; + indicates 1 to 10 colonies; 0 indicates no growth.

table 1. The figures represent the average of ten experiments performed upon nasopharyngeal washings obtained from both adults and children.

Procedure. Shortly after the washings had been collected, a measured amount was placed in a rubber-stoppered Erlenmeyer flask; 10 per cent anesthetic ether was added and the mixture was well shaken. It was then allowed to stand at room temperature for 40 minutes. Dilutions of virus were then made up in samples of these washings. Starting with a 10 per cent suspension of virus (as the "undiluted" sample) further dilutions were carried to 10^{-3} or 10^{-4}. Thus 1 per cent equalled a dilution of 10^{-1}; 0.1 per cent equalled 10^{-2}, etc. These dilutions were made

up in volumes of 10 to 12 cc. and, from each of them, 0.5 cc. was inoculated intracerebrally into one monkey. Thus, in each experiment a titration in tenfold dilutions was run on the unconcentrated samples to define roughly the minimal infective dose.

Concentrating process. The technique was modified slightly during the course of this study (see table 2) but the procedure given below represents that which was eventually adopted and was used in most of the experiments.

In the first experiments the virus suspensions were frozen and dried without the addition of any "protective" substance, but later, normal monkey serum was added. Measured amounts (usually 9.4 cc.) of each of the virus dilutions in nasopharyngeal washings were first placed in 25 cc. tubes suitable for use with the Flosdorf-Mudd apparatus, and to each of them 0.6 cc. of normal monkey serum was added, thus bringing the volume to 10 cc. The material was then frozen in a bath containing dry-ice and methyl cellosolve for 30 minutes. The tubes were then attached to the apparatus, the vacuum pump was started and the evaporating process was allowed to proceed for a period of from four to six hours or until the volume of frozen material in each tube appeared to be not more or not much less than about 0.5 or 0.6 cc.[2] The tubes were then detached and placed in warm water to determine whether the residual material (when melted) had reached the correct volume. (In some instances it was necessary to refreeze some of the samples and continue the evaporation if the volume had not been sufficiently reduced.) With the volume of the concentrate at about 0.5 cc. in each tube, measured amounts of sterile distilled water were added cautiously to each container, sufficient to bring the volume of the concentrate up to exactly 1.0 cc. Dried or sticky material was brought into solution by rubbing the sides of the tube with a sterile capillary pipette. Eventually each concentrate (with a volume of 1.0 cc.) represented a tenfold concentration in volume from the antecedent sample. The series of tubes containing the concentrates

[2] I am indebted to Dr. Henry W. Scherp of the Children's Hospital of Philadelphia for emphasis upon the fact that the suspensions being concentrated should not be evaporated to dryness.

TABLE 2

Comparative virulence titrations in concentrated and unconcentrated nasopharyngeal washings

EXPERIMENT NUMBER	DATE	DEGREE OF EVAPORATION	BACTERICIDAL AGENT	"UNDILUTED" 10 PER CENT	DILUTIONS OF 10 PER CENT VIRUS			
					10^{-1}	10^{-2}	10^{-3}	10^{-4}
1	12/10/36	Unconcentrated	0.5% phenol	0	7			
		Evap. to dryness; conc. 10×	0.5% phenol		—	—		
2	1/ 7/37	Unconcentrated	0.5% phenol	4	5	6		
		Evap. almost to dryness; conc. 10×	0.5% phenol		8	11		
3	2/ 2/37	Unconcentrated	0.5% phenol		—	—	—	—
		Evap. almost to dryness with normal monkey serum; conc. 10×	0.5% phenol			—	—	—
		Evap. almost to dryness with normal monkey serum; conc. 10×	10% ether			—	—	—
4	3/ 1/37	Unconcentrated	0.5% phenol	10	10	—(a)	—(a)	
		Evap. as in exp. 3; conc. 10×	0.5% phenol		8	11*	—	
		Evap. as in exp. 3; conc. 10×	10% ether		8	—	—	
5	3/11/37	Unconcentrated	10% ether		6	—		
		Evap. as in exp. 3; conc. 10×	10% ether		13	5*	12†	
6	4/11/37	Unconcentrated	10% ether		—	—	—	—
		Evap. as in exp. 3; conc. 5–10×	10% ether		16			
		Evap. as in exp. 3; conc. 20–40×	10% ether			17	—	
		Evap. as in exp. 3; conc. 12–60×	10% ether					—
7	6/29/37	Unconcentrated	10% ether	6	—	—	—	—
		Evap. as in Exp. 3; conc. 10×	10% ether		7*	—	—	—

6 = experimental poliomyelitis developed after incubation period of 6 days.

— = no experimental poliomyelitis.

Conc. 10× = theoretical concentration of virus is ten times.

0 = animal died of brain abscess during experiment.

—(a) = animal survived for 28 days with brain abscess.

* Indicates a monkey infected by a concentrate, originally 10× weaker than the M.I.D.

† Indicates a monkey infected by a concentrate, originally 100× weaker than the M.I.D.

were generally allowed to stand in the ice-box overnight in a stoppered container and on the following morning 0.5 cc. from each sample was injected intracerebrally into one monkey. Cultures from these inocula made upon blood agar plates, were always sterile for bacterial growth.

Monkey inoculations. These were done intracerebrally under ether anesthesia, the same volume of inoculum (0.5 cc.) being used in almost all instances (exception experiment 6—table 2). Daily temperature and exercise records were kept on all inoculated animals for a period of four weeks. The length of the incubation period was measured from the day of inoculation to the day of onset of a fever due to experimental poliomyelitis. Thirty-four monkeys were used in the entire series of experiments. Several of the animals which survived without evidences of infection were inoculated more than once.

Results. The experimental procedures are given in table 2. Here it appears that in experiment 1, monkey serum was not added to the dilutions to be concentrated and the evaporation was carried on practically to complete dryness, prior to the material being taken up in small amounts of sterile water. None of the monkeys inoculated with this concentrated material became ill, which was taken to indicate that not only was the virus not concentrated by this procedure but that it did not survive. In experiment 2, again normal monkey serum was not added but the concentrating process was not carried as far. The virus survived this process but the experiment does not indicate whether it was concentrated or not.[3]

In the third experiment none of the inoculated animals came down. The data are included in the table merely to show that the viability of this particular strain and sample of virus was not high and that it was likely to decrease after a few months in glycerol, a feature which, needless to say, emphasizes the importance of doing a virulence titration with each experiment.

In the sixth experiment the attempt was made to increase the

[3] It is worth mentioning in this connection that we have considered variations in the length of the incubation period as a rough indication of dosage.

infectivity of the concentrates still further by carrying the degree of concentration beyond that of ten times.

In experiments 4, 5, 6, and 7, in which monkey serum had been added to the samples to be concentrated, and in which the process was not carried on to complete dryness, there are indications that the virus has been "concentrated" or at least rendered more infective by this process. Primarily, we have in these four experiments, five examples in which the monkey was infected by a concentrate in which the antecedent (unconcentrated) sample was not infective. Omitting experiment 6 (in which the degree of concentration is indefinite and, in which unfortunately none of the control monkeys came down) we find that three monkeys out of four were infected by concentrates derived from samples originally containing ten times less than the minimal infective dose. (See footnote, table 2.) And furthermore in these three experiments one monkey out of five was infected by a concentrate which was derived from a sample originally containing 100 times less than the minimal infective dose. (See experiment 5, table 2.) In experiment 6 there may be a similar indication of enhancement of infectivity as an effect of the concentrating procedures. It seems likely that the result in these last four experiments is more than that of chance.

Clinical trials of method. The method just described has already been applied in the field, on nasopharyngeal washings from three patients from the same family in which a familial epidemic of poliomyelitis was suspected. The virus was successfully isolated from concentrates obtained from two of these three patients.

SUMMARY

In an effort to improve the technique of isolating the virus of poliomyelitis from the nasopharynx in clinical cases of this disease, experiments are described in which the virus has been added to, and recovered from normal nasopharyngeal washings.

Samples of these washings have been concentrated through the agency of freezing and drying in the presence of normal monkey

serum and this procedure has been shown to enhance the infectivity of the virus-containing material.

REFERENCES

FLOSDORF, E. W., AND MUDD, S. 1935 Procedure and apparatus for preservation in "lyophile" form of serum and other biological substances. Jour. Immunol., 29, 389-425.

KRAMER, S. D., SOBEL, A. E., GROSSMAN, L. H., AND HOSKWITH, B. 1936 Survival of the virus of poliomyelitis in the oral and nasal secretion of convalescents. Jour. Exp. Med., 64, 173-182.

PAUL, J. R., AND TRASK, J. D. 1932 The detection of poliomyelitis virus in so called abortive types of the disease. Jour. Exp. Med., 56, 319-343.

PAUL, J. R., TRASK, J. D., AND WEBSTER, L. T. 1935 Isolation of poliomyelitis virus from the nasopharynx. Jour. Exp. Med., 62, 245-257.

TRASK, J. D., PAUL, J. R., BEEBE, A. R., AND GERMAN, W. J. 1937 Viruses of poliomyelitis. An immunological comparison of six strains. Jour. Exp. Med., 65, 687-704.

PHYSICAL CHARACTERISTICS OF CELLS OF AZOTOBACTER, RHIZOBIUM, AND SACCHAROMYCES[1]

HANS LINEWEAVER

Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.

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Recent investigations concerning the mechanism of nitrogen fixation by *Azotobacter* have been concerned with kinetics (Burk, 1934; Endres, 1934a; Lineweaver, Burk and Deming, 1934), essential elements for fixation as distinguished from growth (Burk, 1934; Burk and Horner, 1935b), chemical intermediates and products of fixation (Burk and Horner, 1935a and c; Endres, 1934b and 1935; Roberg 1935; Winogradsky, 1932), and fixation *in vitro* (Bach, Yermolieva and Stepanian, 1934; Roberg, 1936). The present communication reports general physical characteristics of *Azotobacter*, and for comparison, of *Rhizobium* and *Saccharomyces* obtained during direct examination of the enzymic mechanism of nitrogen fixation previously proposed on the basis of kinetic data (Lineweaver, 1938; Lineweaver, Burk and Deming, 1934). The physical characteristics of the cells determined were the density, water content, and relation between dry weight, cell volume, and cell number.

MATERIALS AND METHODS

Azotobacter cells. A. vinelandii, A. chroococcum (Burk strain B8), and *A. beijerinckii* (Burk strain B6) were grown in large scale amounts using methods employed by members of this laboratory working with Dr. G. E. Hilbert. The bacteria were grown at 31°C. in pyrex bottles containing 17 liters of culture medium with 1 or 2 per cent sucrose. The bottles were inoculated with 100 cc. of a 2- to 4-day heavy growth of *Azotobacter*. The cul-

[1] These investigations were carried out in the Biochemical Nitrogen Fixation Section of the Fertilizer Research Division.

tures were aerated with air for 1 to 2 days and then with 1:1 oxygen-nitrogen mixture for 1 to 3 days more. The bacteria were harvested by centrifugation in a Sharples super-centrifuge, the 17 liters of culture yielding from 40 to 90 grams wet weight of bacteria.

For study, the centrifuged cells were re-suspended in culture medium, generally containing 1 per cent sucrose, and consisting of the clear liquid obtained after the following mixture had been thoroughly shaken, allowed to stand, and settle: 0.8 gram of K_2HPO_4, 0.2 gram of KH_2PO_4, 0.2 gram of NaCl, 0.2 gram of $MgSO_4 \cdot 7H_2O$, 0.1 gram of $CaSO_4 \cdot 2H_2O$, 1000 grams of H_2O. Fe as iron humate, about 0.5 mgm. per liter, and Mo as Na_2MoO_4, about 0.1 mgm. per liter, were generally added when the suspension was made. Heat-inactivated cells were prepared by rapidly heating a suspension to 90°C., and, after 5 to 15 minutes, cooling rapidly to 25°C.

Rhizobium meliloti, alfalfa strain 131, University of Wisconsin, was grown by the same technique used for *Azotobacter* and suspended in medium containing 0.2 per cent sucrose.

Baker's yeast cells were obtained from Fleishmann's Yeast Company in commercial starch-free, one pound cakes, and were suspended in medium or in distilled water.

The per cent total solids by weight, was determined by weighing samples before and after drying to constant weight at 110°C. The error was estimated to be about ±0.15 weight per cent.

The densities of the suspensions and centrifugates were determined by weighing in a flask (11.238 cc. at 25°C.) that had a flat ground glass lip for sealing with a ground glass plate. Duplicate determinations generally agreed to within ±0.0004 gram per cubic centimeter.

The cell volumes of the suspensions were determined as illustrated in figure 1 (cf. cell volume of blood, Peters and Van Slyke, 1932, p. 73). The cell suspensions, followed by bromobenzene, were drawn by suction into the lower opening of the tube. The rubber seal, cut from rubber tubing, is adequate since the pressure on the outside of the seal is greater than that on the inside due to the $CaCl_2$ solution. The samples were centrifuged repeatedly at

about 3500 r.p.m. and the per cent volumes read at intervals until they were found to be essentially constant; the total time required was of the order of 30 to 60 minutes.

The dry weight was determined by drying once-washed centrifuged cells to constant weight at 110°C. The error was estimated to be from ±3 to ±5 per cent.

The count, made microscopically, was accurate to ±10 per cent.

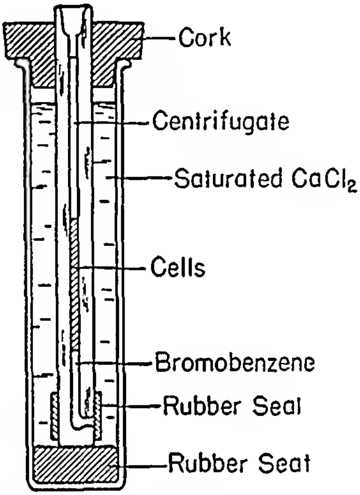

FIG. 1

CALCULATIONS

The water content and total solids by volume of the various liquids are given by the equations:

Water content (grams per cc.) = Density × per cent water by weight ÷ 100

Total solids (grams per cc.) = Density × per cent total solids by weight ÷ 100

The density of the cells and the water content of the cells are given by the following equations:

$$D_c = [D_a - D_b(1 - c.v.)]/c.v., \text{ and}$$

$$\text{Water content of cells (grams per cc.)} = D_c - \text{Dry weight (grams per cc.)} \div c.v.$$

where D_c, D_a and D_b are the densities of the cells, suspension and centrifugate respectively, and *c.v.* is the cell volume in cubic centimeter per cubic centimeter of suspension.

RESULTS AND DISCUSSION

Although an approximately constant relation between cell volume and dry weight per cubic centimeter for any one organism was observed (table 1), there was some slight variation, as indicated in figure 2. This may be attributed to a number of varying factors, such as size of inoculum, rate of aeration, and duration of growth, that are always encountered when growing micro-organisms. The water contents for the cells, reported in table 2, agree with data for various bacteria and yeasts obtained by other investigators upon drying centrifuged cells at 100 to 110°C. (Buchanan and Fulmer, 1928, p. 68). The water content of bacteria generally lies between 75 and 85 per cent (Buchanan and Fulmer, 1928, table VI). The values found for *A. vinelandii* are 80, 78 and 74, for *A. chroococcum* 85, for *A. beijerinckii* 86 and for *R. meliloti* 68 per cent. The two values for the yeast, 79 and 80 per cent, although slightly higher than the average value reported in the literature, 73 per cent, fall well within the wide limits, 68 and 83 per cent.

The density and water content of one species of *Azotobacter*, *A. vinelandii*, were observed to vary between comparatively narrow limits, 1.087 to 1.106, and 74 to 80 per cent. The other two species yielded the values 1.036, 1.04 and 85 and 86 per cent, which appear to be significantly different from those of the first. The explanation for this difference may depend upon the well-known fact that different species produce different amounts of gum-like material, which may intimately surround the cells. Such material would almost certainly have both a different and more variable density and water content than the more opaque cell material.

The absolute accuracy of both the density and water content values, as well as the volume per cell and cell diameter values, depend on the accuracy of the observed centrifugal cell volume, which, in turn, depends on the extent of packing that takes

TABLE 1

Dry weight, cell volume, total solids, density, and water content of experimental material at 25°C.

MATERIAL AND RECORD NUMBER	DRY WEIGHT	PER CENT CELL VOLUME	PER CENT TOTAL SOLIDS	DENSITY Liquid	DENSITY Centrifugate	H_2O CONTENT OF LIQUID
	gram per cc.	*cc. per cc. × 100*	*grams per gram × 100*	*grams per cc.*	*grams per cc.*	*grams per cc.*
Medium			(0.18)	0.9981		0.996
Medium (1 per cent sucrose)			1.173	1.0021		0.990
Azotobacter†						
vin. 60	0.0816	34.0	9.08	1.0326	1.0043	0.939
vin. 64	0.0864	29.5	9.26	1.0333	1.0030	0.938
vin. 68	0.0868	39.8	10.22	1.037	1.0029	0.931
chr. 68	0.0850	56.1	9.73	1.021	1.0015	0.922
beij. 68	0.0850	57‡‡	9.94	1.026		0.921
vin. 46	0.0662	30.1				0.942*
vin. 38	0.0440	18				0.966*
vin. 24-1‡	0.0052	(2.3)††				0.992*
vin. 24-2‡	0.0103	(4.5)††				0.989*
vin. 24-3‡	0.0206	(9.0)††				0.981*
Rhizobium§						
56	0.0874	24.5	9.61	1.0274		0.929
70		2.0		1.0040	1.0020	
Yeast**						
66-1	0.0602	26.3	6.38	1.0182	0.9976	0.953
66-2	0.1701	79.8	18.40	1.0616	0.9999	0.866
42-1	0.0348	15				0.971*
42-2	0.0751	30				0.947*
42-3	0.146	60				0.899*
43-1	0.0459	19				0.965*
43-2	0.1033	40				0.930*
43-4	0.2124	80				0.865*

* Water content (46) = 0.830 (*c.v.*) + 0.990 (1 − *c.v.*) = 0.990 − 0.160 (*c.v.*); water content (38 and 24) = 0.996 − 0.166 (*c.v.*); water content (42 and 43) = 0.996 − 0.164 (*c.v.*); where *c.v.* is the cell volume in cubic centimeter per cubic centimeter.

† The diluent was medium (1 per cent sucrose), exception see footnote ‡.

‡ The diluent was medium (no sucrose).

§ The diluent was medium (0.2 per cent sucrose).

** The diluent was H_2O except for the 42 series where medium was used.

†† The cell volumes of these heat-inactivated cells were calculated by the equation: (*c.v.*) = 435 (dry weight).

‡‡ Somewhat less accurate than other figures, ±3 per cent.

place. If the cells are assumed to be rigid spheres packed horizontally, one sphere directly upon another, 47.64 per cent of the total volume would be filled with water and similarly if the spheres were packed obliquely 25.95 per cent of the space would be filled with water. The cells, however, are seldom exact

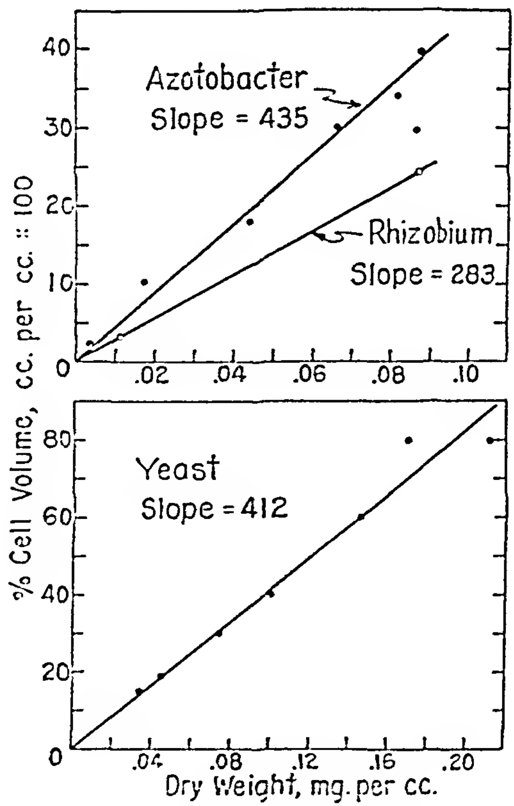

FIG. 2. THE RELATION BETWEEN CELL VOLUME, DETERMINED BY CENTRIFUGATION, AND DRY WEIGHT OF AZOTOBACTER, LEGUME BACTERIA AND YEAST CELLS

spheres and almost certainly are not rigid so that we would not expect a 25 per cent error in the water content figures on this account. Some water will, of course, always be held in the interstices by capillarity, and be a source of error. Another picture of the situation may be obtained from the observation that the volumes per cell calculated from the microscopically estimated

cell diameters were about 50 per cent of the volume per cell obtained by dividing the observed per cent cell volume by the count per 100 cc. (table 2, column 5). These diameters, however, were only about 30 per cent less than those calculated from the observed per cent cell volume (table 2). This order of agreement while not entirely satisfactory, is about as good as can be expected

TABLE 2

Size, density and water content of Azotobacter, Rhizobium and yeast cells at 25°C.

ORGANISM	COUNT	DRY WEIGHT	PER CENT CELL VOLUME	VOLUME PER CELL	CALCULATED DIAMETER	DENSITY OF CELLS	WATER CONTENT OF CELLS	
	millions per cc.	*gram per cc.*	*cc. per cc. × 100*	*c.mm. × 10⁹*	*mm. × 10³*	*grams per cc.*	*gram per cc.*	*per cent by weight*
Azotobacter								
vin. 28-1	10,000	0.0177	10.3	10	2.7			
vin. 28-2	3,200	0.0037	2.2	7	2.4			
vin. 68	30,000	0.0868	39.8	13	2.9*	1.088	0.871	80.0
chr. 68†	3,000	0.0850	56.1	187	7.1	1.036	0.883	85.0
beij. 68†	10,000	0.0850	(57)	(57)	4.8	1.04	0.892	85.8
vin. 60		0.0816	34.0			1.087	0.847	77.9
vin. 64		0.0864	29.5			1.106	0.813	73.5
Rh. meliloti								
33	33,600	0.0114	3.3	1	1.2			
70	12,000		2.0	1.7	1.5	1.10		
56		0.0874	24.5			1.106	0.749	67.6
Yeast (commercial)								
33	7,000	0.199	73.3	105	5.9			
66-1		0.0602	26.3			1.074	0.845	78.8
66-2		0.1701	79.8			1.076	0.863	80.0

* Corresponding ellipsoidal diameters, ratio 1.5 to 1, are 3.4, 2.2.

† These strains contain from 2 to 3 per cent nitrogen while the *A. vinelandii*, under these culture conditions contain 12 to 18 per cent nitrogen.

without detailed microscopic work involving shape and size of the cells, both stained and unstained, as well as counts.

SUMMARY

The relations between dry weight, cell volume, and count per cubic centimeter have been determined for three species of

Azotobacter, for *Rhizobium meliloti* and for baker's yeast. The densities and water contents of the various cells determined were: *Azotobacter vinelandii* cells 1.09 and 0.84 gram per cubic centimeter (average); *Azotobacter chroococcum* 1.04 and 0.87 gram per cubic centimeter; *Azotobacter beijerinckii* 1.04 and 0.88 gram per cubic centimeter; *Rhizobium meliloti* 1.10 and 0.75 gram per cubic centimeter; and *Saccharomyces* 1.075 and 0.85 gram per cubic centimeter.

The writer is much indebted to Dr. Dean Burk for his encouragement and suggestions during the pursuit of this work. He is also indebted to Professor J. C. W. Frazer of Johns Hopkins University for suggestions and criticisms. The valuable criticisms offered by Drs. F. E. Allison and C. A. Ludwig as well as Dr. Burk in the preparation of the manuscript are greatly appreciated.

REFERENCES

Bach, A. N., Yermolieva, Z. V., and Stepanian, M. P. 1934 Fixation de l'azote atmosphérique par l'intermédiaire d'enzymes extraites de cultures d'Azotobacter. Compt. rend. Acad. Sci. U. S. S. R., I, 1, 22-24.

Buchanan, R. E., and Fulmer, E. I. 1928 Physiology and Biochemistry of Bacteria. Baltimore, Vol. I.

Burk, D. 1934 Azotase and Nitrogenase in Azotobacter. Ergebnisse der Enzymeforschung (Nord and Weidenhagen), Leipzig, III, 23-56.

Burk, D., and Horner, C. K. 1935a The production of ammonia by Azotobacter and its relation to the mechanism of nitrogen fixation. Trans. A, Third Intern. Cong. Soil Sci., 1, 148-151.

Burk, D., and Horner, C. K. 1935b The specific catalytic rôle of molybdenum and vanadium in nitrogen fixation and amide utilization by azotobacter. Ibid., 1, 152-155.

Burk, D., and Horner, C. K. 1935c Über Hydroxylamin, Hydrazin und Amide als Intermediärprodukte bei der N_2-Fixation durch Azotobacter. Naturwiss., 23, 259-260.

Endres, G. 1934a Zur Kenntnis der stickstoffassimilierenden Bakterien I. Ann. Chem. I, 512, 51-80.

Endres, G. 1934b Über ein Zwischenprodukt der N_2-Assimilation. Naturwiss., 22, 662.

Endres, G. 1935 Zur Kenntnis der stickstoffassimilierenden Bakterien II Über die Bindung des Luftstickstoffes durch Azotobacter. Ann. Chem. II, 518, 109-126.

Lineweaver, H. 1938 The Solubility and Chemical and Physical Absorption of Nitrogen Gas in Azotobacter Cells. Jour. Biol. Chem., **122**, 549-567.

Lineweaver, H., Burk, D., and Deming, W. E. 1934 The Dissociation Constant of Nitrogen-Nitrogenase in Azotobacter. Jour. Amer. Chem. Soc., **56**, 225-230.

Peters, J. P., and Van Slyke, D. D. 1932 Quantitative Clinical Chemistry. Baltimore, **2**,

Roberg, M. 1935 Beiträge zur Biologie von Azotobacter. II. Der Stickstoffgehalt der Filtrate von Azotobakterkulturen. Jahrb. f. Wiss. Bot., **82**, 65-98.

Roberg, M. 1936 Beiträge zur Biologie von Azotobacter. III. Zur Frage eines ausserhalb der Zelle den Stickstoff bindenden Enzyms. Jahrb. f. Wiss. Bot., **83**, 565-592.

Winogradsky, S. 1932 Sur la synthèse de l'ammoniaque par les Azotobacters du sol. Ann. Inst. Pasteur, **48**, 269-301.

DISSOCIATION OF SINGLE CELL CULTURES OF STAPHYLOCOCCUS AUREUS[1]

RACHEL E. HOFFSTADT AND GUY P. YOUMANS[2]

Department of Bacteriology, University of Washington, Seattle

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In 1932, a virulent strain of *Staphylococcus aureus* was dissociated by us into eight rough dissociants and one "G" strain. Later in 1934, we studied five other strains of *Staphylococcus*, of which three were classified as *S. aureus*, and two as *S. albus*. From these five cultures, eight stable strains were isolated which varied physiologically from the parent culture.

This study of single cell strains was undertaken in order to confirm results previously obtained by using colony selection as a method of purifying the cultures.

METHODS

Thirty-three single-cell isolations were made from the smooth undissociated form of *Staphylococcus aureus*, using a Chamberlain micromanipulator, and growth was obtained from only two of these. These strains were designated as numbers 24 and 31. One active single-cell culture was secured from a white rough variant of the same culture.

The culture from which these cells were obtained was the one used by us in all previous studies on dissociation. It fermented maltose, mannitol, sucrose and glucose, but did not ferment lactose, inulin, salicin or galactose. Both strains 24 and 31 and the single culture of the rough variant, were planted on plain broth and lithium chloride broth with a pH of 7.8, and transplanted daily for forty days. Agar plates were made at each transplant to determine the amount and type of dissociation produced.

[1] This work was aided by a grant from the Research Committee of Sigma Xi.

[2] Now Instructor of Bacteriology, Northwestern Medical School, Chicago.

RESULTS

Strains 24 and 31 were found to differ culturally from the parent strain in that they fermented glycerol.

During the experiment, strain 24 produced two rough yellow variants, but no white ones. The first (RY_1), appearing on the 16th day, retained the biochemical characteristics of the parent strain, but gave a distinctly rough granular appearance on agar and agglutinated spontaneously in broth.

The second variant (RY_2) which appeared after the 21st transfer displayed the same cultural characteristics as the first, except that it fermented galactose. In this it also differed from the

TABLE 1

Fermentation reactions of single-cell strains 24 and 31 and their variants

MEDIA	24	31	RY_1	RY_2	RW_1	RW_2	G
Glucose	+	+	+	+	+	+	–
Sucrose	+	+	+	+	+	+	–
Maltose	+	+	+	+	+	+	–
Lactose	–	–	–	–	+	+	–
Salicin	–	–	–	–	–	+	–
Glycerol	+	+	+	+	+	+	–
Galactose	–	–	–	+	+	+	–
Mannitol	+	+	+	+	+	–	–
Inulin .	–	–	–	–	–	–	–
Litmus milk .	–	–	–	–	+	+	–
Source	SY	SY	21	21 & 31	31	31	31

parent strain. Both reverted to a smooth yellow growth on agar on subsequent culturing and both differed from all yellow variants previously described by us.

Single cell culture 31 dissociated more than strain 24 (table 1). A rough yellow variant appeared on the 23rd day which was similar to the yellow rough variant obtained from strain 24 on the 16th day. In addition, after the 4th day one light yellow variant, RW_1, developed which differed from the parent culture in that it fermented lactose, galactose and formed acid in litmus milk. This variant on successive days became lighter in color until the 31st day when it became completely white (RW_2); it then fer-

mented salicin as well as lactose and galactose. The variant RW_2 was the only one similar to any dissociant previously obtained by us which was isolated once before from an entirely different source. Neither culture dissociated as readily as did the culture when originally studied.

The G form was found only once. It was obtained from strain 31 on the 31st transfer and only fermented galactose. It is interesting to note that the enzyme fermenting galactose apparently is the first in this case which appears to become active upon dissociation. The dissociation of strain 31 was more pronounced in plain broth than in lithium chloride broth and the reverse was true with strain 24.

Antigenic studies were made in terms of cross agglutination of strain 31, RY_1 (yellow rough obtained from 24), RY_2 (yellow rough obtained from 24 and 31), RW_1 and RW_2 (white roughs from 31). The antisera against these organisms were produced by the inoculation of rabbits to which were given three doses of living organisms at weekly intervals. Although sera produced by strain 31 showed agglutinins for all variants obtained from both strains 31 and 24, the titers were much lower than in any of the sera produced from the variants of either strain. Strain RY_2, the yellow rough variant obtained from both 31 and 24, showed the most nearly uniform reaction to all sera, while RW_1 and RW_2 showed a lower titer for the RY_1 and RY_2 and 24 sera than they did in RW_1 and RW_2 sera. None of them produced agglutinins or reacted with the G sera except RW_1.

A very suggestive observation was made during the study of the more actively dissociating strain 31. The first visible change which took place was a change in pigment production,—orange to cream to white. The thin granular rough appearance of the colony appeared during this time. When the pigment was a light yellow the enzyme which fermented galactose became active in the RY_2 strain. It was also more active antigenically than the parent 31 strain.

The fourth change was the increased activity for the lactose-fermenting enzyme and lastly the salicin-fermenting one. Increased antigenic activity was observed during this period. The

results seem to confirm the results previously reported by us that there are three to four antigenic factors in the undissociated organism which become active on dissociation. None of the variants were virulent. A single cell study of a stable white variant which was obtained from the original culture was made, but apparently it had become stable because no changes were observed.

SUMMARY

Both single-celled strains obtained from the whole-yellow undissociated strain of *Staphylococcus aureus* dissociated, while the single cell strain obtained from the white variant of this same culture did not dissociate. There was a difference in the capacity to dissociate between strain 31 and strain 24. The former produced both white and yellow variants while the latter only produced yellow; the former dissociated more readily in plain broth, while the latter dissociated only in lithium chloride broth. A G form was obtained only from strain 31. All white forms were more active enzymatically than the yellow forms, and the sera produced from all strains showed agglutinins for all strains. None of the strains were virulent, and only one strain obtained in this study had been reported by us before and none gave the cultural characteristics of the type strains usually described.

From the above results the variation of the physiological characteristics of bacteria upon dissociation, would seem to be profoundly influenced by the type of cell dominant at the time of culture.

REFERENCES

Hoffstadt, R. E., and Youmans, Guy P. 1932 *Staphylococcus aureus*. Dissociation and its relation to infection and to immunity. Jour. Inf. Dis., 51, 216-242.

Hoffstadt, R. E., and Youmans, Guy P. 1931 The genetic significance of the dissociants of *Staphylococcus aureus*. Jour. Bact., 27, 551-560.

THE INFLUENCE OF DIET ON THE L. ACIDOPHILUS CONTENT AND H-ION CONCENTRATION OF THE INTESTINE

LOUIS WEINSTEIN, JAMES E. WEISS AND RUSSELL W. H. GILLESPIE

Department of Bacteriology, Yale University, New Haven, Connecticut

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In the course of their extensive experiments on intestinal flora Rettger and Cheplin (1921) came to the conclusion that lactose has a modifying effect on the bacterial inhabitants of the colon because it acts as food for the organisms, and not because of any depressing effect which it exerts on the H-ion concentration of the contents of the intestine. Some investigators have questioned this hypothesis and claimed that a direct correlation could be established between the numbers of *Lactobacillus acidophilus* present in the intestine and the H-ion concentration. They concluded that lactose changes the type of intestinal bacterial population by depressing the pH to the point where *Escherichia coli* is inhibited and the aciduric organisms become the predominant type.

For literature on this subject the reader is referred to the annotated bibliography of Frost and Hankinson (1931). One of the first publications which appeared to controvert the conclusions drawn by Rettger and Cheplin was that of Cannon and McNease (1923). These workers studied the effect of diet on the bacterial types and on the H-ion concentration of the colon of rats, and came to the following conclusion: "The simplification of the intestinal flora varies directly with the H-ion concentration, a pH of 7.0 being characteristic of a gas-producing proteolytic type, whereas an increasing acidity is characterized by a diminution of proteolytic types and their replacement by aciduric types mainly dominated by *L. acidophilus*." Cannon and McNease believed that the slow decomposition of lactose, which

Rettger and Cheplin were able to demonstrate and which they claimed is responsible for the ability of *L. acidophilus* to increase in numbers in the intestine, suggests the possibility of a more favorable pH environment for the aciduric types. Kendall (1921) was in accord with the observations of Cannon and McNease; he claimed that an available carbohydrate is acted upon by both proteolytic and fermentative types of organisms, and that as a result of this an acidity develops which is distinctly unfavorable to the former group.

It was our purpose in the work presented here to repeat the experiments of Rettger and Cheplin and of Cannon and McNease, using more types of diets, studying the bacterial flora and the pH of the entire intestine, instead of just the colon, and using the more exact method of H-ion concentration determination which has been developed since the above workers published their results. A medium better suited for the growth of *L. acidophilus* than that used by either of the above groups of investigators was also available. In short, the work was done primarily to determine whether any direct correlation can be established between the presence of the aciduric bacilli and a high H-ion concentration, or whether *L. acidophilus* can increase in numbers in the intestine merely as the result of a specific type of favorable diet, without being influenced by the pH of the intestinal contents.

EXPERIMENTAL

Mature white rats were used because of their ready availability and the ease with which their intestinal flora can be influenced by diet. The following diets were employed, the amounts indicated below being fed daily.

1. 12 grams of chopped lean beef.
2. 12 grams of chopped lean beef plus 3 grams of lactose.
3. 10 grams of "calf-meal." This mixture contains 15 per cent linseed oil, 20.75 per cent corn meal, 10 per cent ground malted barley, 22 per cent wheat flour, 12 per cent dried skimmed milk, 15 per cent oat flour, 3 per cent soluble blood flour, 1 per cent salt, 1 per cent steamed bone meal and 0.25 per cent cod liver oil reinforced with vitamin D.

Sixty rats were divided into three groups. One group of twenty was fed diet 1 for three weeks, then sacrificed and the bacteria and pH of the various segments of the intestinal tract determined. Twenty rats were fed diet 2 for three weeks, and twenty diet 3 for three weeks, and the animals subjected to the same treatment. With the second set of rats a preliminary feeding of ration 1 for two weeks was resorted to before diet 2 was fed.

The rats were killed with illuminating gas, and the abdomens opened immediately after death. Portions of the duodenum, jejunum, upper ileum, lower ileum, cecum, upper colon, middle colon and lower colon were removed aseptically. The contents were stripped into physiological saline solution and portions of the suspensions plated in media which are particularly suitable for the detection of *L. acidophilus*. The methods used for culturing the intestinal material have been described previously by Weinstein, Weiss, Rettger, and Levy (1933).

The H-ion concentration of the material taken from the various levels of the intestinal tract was determined by means of glass electrodes accurate to within 0.02 unit pH. Small, bulb-type electrodes (6 to 9 mm. in diameter) prepared in the laboratory by one of us (R. W. H. G.) were used throughout this work. The electrodes were made by immersing platinum wire in N/10 HCl-quinhydrone solution within the bulb. Phthalate buffer, pH 3.97, was used as the primary standard against which the glass electrode was frequently checked. Only electrodes which repeatedly checked against buffers with less than 0.02 pH error were used.

The intestinal material was obtained in quantities ranging from 0.1 to 1.0 cc. or more. The nature of this material and the method used for the determination of pH necessitated dilution with from one to three or four (rarely five) parts of distilled water. It was established that dilution to this extent introduced no significant error. The test material was mixed thoroughly with the distilled water, the mixtures placed in small specially prepared vessels and the glass electrode potentials determined with the use of the saturated calomel electrode for reference and the circuit devised by Du Bois (1930), for determining potential

differences. Laboratory temperature was observed and the proper factor used in calculating pH values. The error in the determination of H-ion concentration was probably, with few exceptions, well within the pH limit of 0.05.

TABLE 1

H-ion concentration and L. acidophilus content of various portions of intestine of animals fed on a meat diet

RAT NUMBER	DUODENUM		JEJUNUM		UPPER ILEUM		LOWER ILEUM		CECUM		UPPER COLON		MIDDLE COLON		LOWER COLON	
	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.
1		0	6.17	0	6.67	80	7.80	0	6.89	0			6.01	0	6.11	0
2		0	6.09	0	6.52	85	7.99	60	6.41	50					6.39	0
3		0	6.43	0	6.81	0	7.30	80	7.01	30					6.92	0
4		0	6.39	0	6.79	0	7.17	0	6.61	0					6.47	0
5		0	7.17	0	6.92	0	7.70	15	7.23	5			6.71	0	6.46	0
6	6.35	0	6.37	0	6.59	0	7.71	0	6.51	0			5.99	0		
7	6.55	0	6.39	0	6.49	0	6.87	0	6.70	0			6.65	10		
8	6.65	0	6.55	0	6.53	0	6.72	0	6.98	0	6.31	0	6.60	0	6.72	0
9	6.42	0	6.29	0	6.25	0	6.59	0	6.78	0			6.77	0		
10	6.42	0	6.47	0	6.51	0	6.70	0	6.39	0	6.36	0	6.13	0		
11	6.26	0	6.31	0	6.95	50		0	6.75	30						
12	6.30	0	6.37	0	7.21	0	7.51	0	6.95	15						
13	6.43	90	6.75	90	6.69	90	6.89	90	7.26	0	6.28	0	6.13	0		
14	6.48	0	6.26	0	6.73	0	7.43	0	7.16	0			6.85	0		
15	6.39	0	6.36	0	6.58	0	6.91	0	7.26	0			6.72	0		
16	6.63	0	6.57	0	6.90	0	7.41	0	6.71	0						
17	6.51	0	6.80	0	6.55	0	7.03	0	6.93	0			6.33	0		
18	6.58	0	6.60	0	6.73	0	7.27	0	7.59	0			7.18	0		
19	6.53	0	6.27	0	6.45	0	6.91	0	6.87	0	6.83	0	6.75	0	6.71	0
20	6.51	0	6.33	0	6.71	0	7.55	0	6.99	0	6.88	0	6.69	0		

RESULTS

Meat diet

The pH readings and bacteriological findings on the animals which were fed a meat diet for three weeks are given in table 1. Over a pH range of 6.30 to 6.65 *L. acidophilus* was recovered only once from the duodenum; the pH in this instance was 6.43, and the aciduric organisms constituted 90 per cent of the total viable flora.

The H-ion concentration of the jejunum of animals on a meat diet was found to vary between 6.09 and 6.80. *L. acidophilus* was present in only one instance, at a pH of 6.75.

The H-ion concentration of the upper ileum of meat-fed animals ranged between pH 6.45 and 7.24. *L. acidophilus* was demonstrable in the ileum in four of the twenty animals, at H-ion concentrations of pH 6.52, 6.67, 6.69 and 6.95. The number of *L. acidophilus* cells present varied between 50 and 90 per cent.

The pH of the lower ileum, that is the portion immediately distal to the cecum, was found to lie between 6.59 and 7.99. *L. acidophilus* was demonstrated in four animals at H-ion concentrations of 6.89, 7.30, 7.70 and 7.99. It is very interesting to note that the aciduric intestinal organism was present in amounts as high as 60 and 80 per cent at pH levels of 7.99 and at 7.30.

The H-ion concentration limits of the ceca of the animals which had subsisted on a meat diet were pH 6.39 and 7.59. *L. acidophilus* was found in five of the animals, at H-ion concentrations varying from 6.78 to 7.23.

L. acidophilus was recovered only once from the colon of the twenty rats used in this experiment, namely in an animal the pH of whose cecum was 6.65. Here it constituted 10 per cent of the total viable intestinal flora. The H-ion concentrations of the colons of the twenty animals used were found to range between 5.99 and 7.18.

Meat and lactose diet

The H-ion concentration and bacteriological findings for animals given a meat and lactose diet for three weeks are presented in table 2. The pH of the duodenum of these animals varied between 6.04 and 6.72. *L. acidophilus* was recovered in all but 8 of the 20 animals.

The pH of the jejunum of animals fed the same diet ranged between 5.76 and 6.65. *L. acidophilus* was demonstrable in all but 9 of the animals used.

The H-ion concentration of the upper ileum of the rats which subsisted on a diet composed of meat and lactose varied between

5.07 and 7.24. The aciduric intestinal organism was found in all but three of the animals.

The pH of the lower ileum of the rats kept for three weeks on the same diet varied between 5.08 and 7.87. *L. acidophilus* was present in eighteen of the twenty animals studied.

TABLE 2

H-ion concentration and L. acidophilus content of various portions of intestines of animals fed on a meat diet plus 8 grams lactose

RAT NUMBER	DUODENUM		JEJUNUM		UPPER ILEUM		LOWER ILEUM		CECUM		UPPER COLON		MIDDLE COLON		LOWER COLON	
	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.
1	6.68	0	6.31	0	6.76	0	6.63	0	6.11	0			6.22	0	6.38	0
2	6.48	0	6.28	0	7.21	5	6.26	95+	6.43	95	5.73	95+			6.31	95+
3	6.18	0	6.05	0	6.13	0	6.17	0			6.28	0			6.27	0
4	6.27	0	6.14	0	5.77	70	5.83	85	6.03	75			6.31	95		
5	6.43	0	5.20	0	5.93	50	5.84	10	6.12	95+	5.56	95+	6.19	95+	6.39	95+
6	6.04	0	6.36	0	5.31	0	5.08	0	4.61	85					4.84	90
7	6.08	40	5.97	40	6.09	60	5.99	80	5.86	90	6.05	95	6.26	95		
8	6.19	90	5.85	95	5.11	95	5.09	95	5.12	95	5.12	95	5.36	95	5.29	95
9	6.12	0	6.00	0	6.31	90	5.14	95	4.73	95	5.52	95	5.39	95	5.13	90
10	6.18	10	5.76	0	6.12	30	5.91	90	5.90	50	5.41	90	5.97	80	6.12	80
11	6.51	60	6.53	95	5.59	95	5.08	95	6.71	95			6.39	95		
12	6.53	85	6.53	95	6.97	95	6.12	95	6.95	95	6.55	95	6.41	95		
13	6.23	90	6.45	85	5.07	85	5.41	95	6.53	85	5.06	90	5.57	95	5.60	90
14	6.37	40	6.36	60	5.95	70	6.75	70	6.53	95	5.43	95	5.75	90	5.60	90
15	6.20	30	6.11	50	6.04	50	6.83	60	6.31	90	6.37	90	6.26	90	6.19	90
16	6.52	95	6.82	95	6.95	95	7.16	95	6.66	95	6.45	95			6.41	95
17	6.24	70	6.29	80	5.36	95	5.11	95	4.83	95	6.21	95			6.17	95
18	6.27	95	6.50	95	5.67	95	5.41	95	5.65	95			6.50	95	6.01	95
19	6.59	50	6.65	0	7.05	60	7.87	10	7.28	95			5.99	95		
20	6.72	0	6.46	50	6.67	15	6.74	30	7.63	75	7.26	75			6.67	75

The H-ion concentration of the ceca of these rats was found to range from pH 4.64 to 7.63. All but one of the animals studied harbored very large numbers of *L. acidophilus* in the cecum.

The pH of the colon of the rats given a meat and lactose diet varied between 4.84 and 7.26. *L. acidophilus* was present in all but 2 of the animals studied; it could be demonstrated in high amounts at a pH as high as 7.26.

Calf-meal diet

This diet was fed to animals because it was thought to approximate most closely the type of diet which rats normally eat. The H-ion concentration and bacteriological findings of the intestinal contents of these animals are given in table 3.

TABLE 3

H-ion concentration and L. acidophilus content of various portions of intestine of animals fed on a calf-meal diet

RAT NUMBER	DUODENUM		JEJUNUM		UPPER ILEUM		LOWER ILEUM		COLON		UPPER COLON		MIDDLE COLON		LOWER COLON	
	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.	pH	% Ac.
1		0		0	8.55	0	9.34	0	5.67	25			5.69	15	6.52	40
2		0	6.67	0	7.80	0	8.45	0	5.70	80			5.90	75		
3		0	6.41	0	6.99	0	8.77	0	5.91	10						
4		0		0	7.77	50	9.09	50	6.17	80			6.00	85	6.69	85
5		0		0	7.80	0		0	6.21	50					6.47	65
6		20			7.32	50	7.92	0	6.89	95			6.17	40		
7		0	6.78	0	6.83	0	7.63	50	7.01	40			6.97	40	8.54	40
8		0	6.63	0	7.65	0	8.93	0	6.03	0			6.31	0	8.09	20
9		0	5.87	0	6.82	0	8.42	0	6.25	20			6.44	25	8.45	10
10		0	6.54	0	8.41	60	8.79	80	6.08	95			5.96	90	6.53	85
11		10	6.43	40	6.78	80	7.31	90	6.75	90			6.31	95	6.69	95
12		60	6.08	35	6.62	90	8.09	90	5.82	95			6.63	90	6.64	90
13		50	6.59	0	6.92	85	8.89	85	6.23	85			6.22	90	6.62	10
14		90	6.35	95	6.63	70	6.89	65	6.52	90			6.59	95	5.92	85
15		5	6.39	10	7.08	90	8.48	95	6.65	95			6.20	50	8.68	80
16	6.17	60	6.37	70	7.13	60	8.33	90	5.69	90+			5.76	95	6.07	95+
17	6.07	50	6.09	39	5.49	60	5.23	80	6.23	90			5.51	95	5.81	95
18	5.98	0	5.98	0	6.68	20	8.33	0	6.06	0			5.93	0	6.34	0
19	5.99	20	5.72	40	5.30	60	5.46	60	5.99	85			5.62	95+	5.72	95+
20	6.10	10	6.10	30	5.51	25	5.44	80	5.90	95+			5.70	95+	5.54	95+

Determinations of the pH of the duodenal secretions were made in only 5 of the twenty animals studied, due to lack of sufficient material in the other rats. The pH varied between 5.98 and 6.17. *L. acidophilus* was present in ten of the twenty rats in concentrations varying from 5 to 90 per cent.

The H-ion concentration of the jejunum of these animals ranged between 5.72 and 6.78. Only eight of the rats studied harbored *L. acidophilus* in this portion of the small intestine.

Determinations of the H-ion concentration and *L. acidophilus* content of the upper ileum of the rats receiving the calf-meal diet showed a pH range of from 5.30 to 8.55. *L. acidophilus* was present in all but six of the animals.

The pH of the lower ileum varied from 5.23 to 9.34. Eight of the 20 animals were found to harbor no *L. acidophilus* in this portion of the intestine.

All but two of the 20 rats which had subsisted on a calf-meal diet had *L. acidophilus* in the cecum. The H-ion concentration of this portion of the small intestine was found to lie between pH 5.67 and 7.01.

The pH of the colon of the animals fed the calf meal diet ranged between 5.51 and 8.45. Even at the high pH level (8.45) *L. acidophilus* constituted 40 per cent of the total viable intestinal flora. The organism was found to be present in the colon of all but two of the animals studied.

DISCUSSION

A close perusal of the tables will show that in many instances there is absolutely no correlation between H-ion concentration and the presence or absence of *L. acidophilus* in the various portions of the intestine of white rats fed on the diets described above. Frequently this aciduric organism was present at pH levels which were much higher than those at which no organisms of this type could be detected. Indeed, in a number of instances it was found to be present at H-ion concentrations which were definitely on the alkaline side.

In table 4 are presented *average* figures for the H-ion concentration determinations and percentages of *L. acidophilus* found in various segments of the intestine of rats kept on different diets. It will be seen here that there is very little correlation between pH and the occurrence of *L. acidophilus* in the respective segments of the small intestine. For example, in rats having the calf-meal diet, the *L. acidophilus* incidence was much greater in the lower ileum at a pH of 7.88 than in the duodenum at a pH of 6.45, or the jejunum at pH 6.31. While *no correlation between* H-ion concentration and *L. acidophilus* content appeared to exist

in the small intestine, a degree of relationship between pH and the amount of *L. acidophilus* present appeared to exist in the large intestine, that is from the cecum to the rectum. In tables 1 to 3, however, there are a number of instances in which correlation in the large intestine also falls down. Thus, in some individual animals, *L. acidophilus* was found present at pH levels

TABLE 4

Averages of the pH and L. acidophilus content of various portions of the intestine on various diets

	MEAT	MEAT + LACTOSE	CALF-MEAL
Duodenum:			
pH	6.47	6.31	6.45
% Acid	0	38	19
Jejunum:			
pH	6.39	6.28	6.31
% Acid	5	42	19
Upper ileum:			
pH	6.63	6.06	7.00
% Acid	13	60	40
Lower ileum:			
pH	7.18	6.05	7.88
% Acid	12	66	48
Cecum:			
pH	6.90	6.17	6.18
% Acid	7	80	65
Upper colon:			
pH	6.53	5.90	5.52
% Acid	0	93	91
Middle colon:			
pH	6.57	6.07	6.11
% Acid	1	84	65
Lower colon:			
pH	6.54	6.19	6.75
% Acid	0	84	64

which were definitely alkaline. Comparison of the H-ion concentration of the intestines of rats fed different diets reveals that *L. acidophilus* was present in many instances at a pH of 6.0 to 6.8, while in some it was absent at a pH much below this level. In general, however, both on the basis of the figures for each individual animal on the various diets and of the average

figures as presented in table 4, there appears to be a more or less definite relationship between the pH and the acidophilus content of the colon.

Analysis of the data in table 4 seems to indicate that the type and complexity of the diet fed play some rôle in determining the H-ion concentration and bacterial flora of the intestine. Thus, the calf-meal diet which contains lactose and starch, both of which are known to be flora-altering carbohydrates, led to the production of secretions having a considerably lower H-ion concentration than the meat diet which contains no known flora-altering carbohydrates. The level of the intestine at which the pH and bacterial population are studied also seems to play some rôle in determining whether or not a correlation can be established. For example, the average per cent of *L. acidophilus* in the duodenum and jejunum was low even when lactose-containing diets were fed.

CONCLUSIONS

In an attempt to determine whether or not a definite correlation could be established between the H-ion concentration and bacterial population of the various portions of the intestine in rats which were fed diets consisting of lean chopped meat, meat with added lactose, and "calf-meal" the following results were obtained.

1. No correlation between pH and *Lactobacillus acidophilus* content was demonstrable in the small intestine. In the colon, however, there was some degree of correlation between the H-ion concentration and the amount of *Lactobacillus acidophilus* present. While in various individual instances correlation was not evident, it was apparent when the interpretations were based on average figures.

2. The nature of the diet used and the portions of the intestine examined are of seeming importance in the determination of the average pH and numbers of *Lactobacillus acidophilus* found. These two factors must be taken into consideration in establishing the type of correlation attempted here.

REFERENCES

Cannon, P. R., and McNease, B. W. 1923 Factors controlling intestinal bacteria. Influcnce of H-ion concentration of bacterial groups. Jour. Infect. Dis., 32, 175–180.

Du Bois, D. 1930 A vacuum tube potentiometer applicable for use with glass electrodcs of high rcsistance. Jour. Biol. Chem., 88, 729–741.

Frost, W. D., and Hankinson, H. 1931 Lactobacillus Acidophilus (an annotated bibliography to 1931). David-Greene Corporation, Milton, Wisconsin.

Kendall, A. I. 1921 Textbook of Bacteriology.

Rettger, L. F., and Cheplin, H. A. The Transformation of Intestinal Flora, with Special Rcference to the Implantation of Bacillus acidophilus. Yalc University Press, New Haven, 1921.

Weinstein, L., Weiss, J. E., Rettger, L. F., and Levy, M. N. 1933 The therapeutic application of acidophilus milk in simple constipation. Arch. Int. Mcd., 52, 384–397.

METABOLISM OF PATHOGENIC BACTERIA

I. Bacteriological and Chemical Methods[1]

THEODORE E. FRIEDEMANN[2]

Department of Medicine, University of Chicago, Chicago

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The study of the metabolism of pathogenic bacteria presents certain difficulties not frequently encountered with non-pathogenic saprophytic organisms. The chief difficulty is the inadequacy of many analytical methods when applied to the extremely complex culture media often required for the growth of certain pathogens. More specific methods are needed for such media. Some organisms yield only very small quantities of certain metabolic products, such as lactic acid, alcohol, or volatile acids. Again, it may be desirable to carry out a complete analysis on a medium, such as serum, of which a relatively small quantity is available. In order to apply to small quantities of metabolic products or to small samples, more sensitive methods must be used. Finally, there is the hazard of infection, which may to some extent account for the fact that the metabolism of pathogenic bacteria has not been investigated as thoroughly as that of the non-pathogens.

Since the initiation of this work in 1929 a technique for safely handling virulent cultures has been developed and more selective and sensitive methods for the determination of the chief metabolic products, lactic acid, alcohols, and volatile acids have been devised (Friedemann, Cotonio and Shaffer, 1927; Friedemann, 1928; Friedemann and Graeser, 1933; Friedemann and Klaas, 1936; Friedemann, 1938). These, and other methods necessary

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[2] With the technical assistance of Mr. Theodore Brook.

for a more complete analysis, will be briefly described in this paper. Metabolic data will also be presented in order to demonstrate the accuracy of the methods and consistency of the results as a whole. Discussion of the metabolic data will be deferred to a later paper.

BACTERIOLOGICAL METHODS

The medium was freshly prepared before each experiment. In the majority of experiments reported in this and subsequent papers, this consisted of 10 volumes of sterile meat infusion (with 1 per cent peptone and from 0.3 to 0.75 per cent anhydrous Na_2HPO_4, initial pH 7.8), 1 volume of sterile 11 per cent glucose and 2 per cent by volume of serum. The medium was thoroughly mixed to insure homogeneity. Accurately measured volumes were then transferred to sterile tall dispensing bottles. A measured volume of inoculum, or a loop-full of organisms from solid medium, was then added. Incubation was in a water-bath regulated at 37.5°C.

Growth was stopped instantly by the addition of exactly 10 cc. of N H_2SO_4 for each 100 cc. The gauze-cotton plug was replaced. In the case of gas-producing organisms the bottles were rotated frequently for 30 to 60 minutes to permit escape of gases. The plug was then discarded (it was sterilized) and replaced by a hot paraffined cork stopper; the neck of the bottle was sterilized by a brief immersion in hot paraffin. The acidified culture was kept in the refrigerator until ready for analysis.

Acidification to approximately 0.1 normality is sufficient to stop growth of most organisms instantly. Sterility is attained with some within an hour. Spore-bearing bacteria and pathogenic yeasts and molds are not killed by the acid, although their metabolic activities appear to be virtually stopped. However, addition of a very small quantity of $HgCl_2$ with the acid readily sterilizes yeast and mold cultures. Acidified cultures have been kept at room temperature for some time, and frequent samples have been withdrawn without sterile precautions, with no apparent growth of contaminating organisms.

At this acidity the concentration of many of the metabolic

products remains unaltered over a long period of time. Determinations of alcohol, volatile acids, lactic acid, and glucose in cultures of the pneumococcus, *Escherichia coli*, and *Eberthella typhosa*, made 60 to 100 days after acidification, agreed, within the limits of experimental error, with those made immediately after acidification. *Polysaccharides and proteins are slowly hydrolyzed by the acid.*

While the bacteriostatic and bactericidal action of acids has long been known (Winslow and Lochridge, 1906; Cohen, 1922), acids have not been used to any extent in metabolic work. Acidification is superior to filtration (Hewitt, 1932a) and to heating (Harden, 1901) since it stops metabolic activities instantly and no precautions need be taken to prevent further contamination. Filtration is not without danger of accidental infection, since it may involve transfer of large volumes of the culture from the flask to the filter. Loss of volatile constituents and decomposition of labile organic compounds is negligible in acidified cultures. Perhaps the greatest source of error resulting from sterilization by filtration or heat is from the large and variable moisture loss, which cannot be entirely corrected by blanks. The latter error is avoided by acidification.

Blanks

One or more of the inoculated bottles are immediately acidified. Since all bottles initially contain the same constituents, any increase or decrease in the incubated bottles is considered to be due to metabolic activities of the organism.

CHEMICAL METHODS

Reducing sugar (glucose)

Proteins, "saccharoids," and other interfering substances are removed by the Somogyi (1930) method. Five cubic centimeters of the acidified medium are transferred to a volumetric flask. Ten cubic centimeters of a 10 per cent $ZnSO_4 \cdot 7H_2O$ solution, water, and a few drops of 1 per cent phenolphthalein are added. Approximately N NaOH is then added until a faint pink color persists. If the culture medium is colored, the volume of N

NaOH necessary for neutralization of the $ZnSO_4$ solution is added, plus an additional volume to neutralize approximately the acidity of the aliquot. The volume is then brought to the mark and the filtrate is analyzed in quadruplicate by the Shaffer-Hartmann (1920–21; Shaffer and Somogyi, 1933) method. A part of the filtrate is saved for the determination of lactic acid.

If the sugar content is less than 20 mgm. per 100 cc., a known quantity of glucose should be added just before precipitation. For media which contain little protein the zinc precipitation may be omitted if the quantity of sugar is such as to require a dilution greater than 1 to 50.

The copper reagent with KI (Shaffer's reagent 50) is preferred, since it appears to yield more constant and consistent results. It should be checked at frequent intervals against a culture medium to which have been added known quantities of glucose and which has been precipitated and diluted exactly as the acidified cultures.

Precipitation of media with lead salts has been suggested by Stiles, Peterson, and Fred (1926). The writer finds, however, that adequate removal of interfering substances requires a larger amount of lead than is recommended by these authors, and low results are often obtained. These observations thus agree with those of Magee and Smith (1930). The Somogyi procedure applied to culture media provides excellent removal of interfering materials and furthermore possesses the distinct advantage of removing no glucose.

Hydrolyzable sugar

Two 2-cc. samples (of serum) are allowed to drain slowly from the pipette into large test tubes which are calibrated at 50 cc. Five cubic centimeters of 2 N H_2SO_4 are added. The tube is covered either with a 1-hole rubber stopper or with a glass bulb. The contents are incubated 4 hours, *with frequent mixing*, in a boiling water bath. They are then cooled. Ten cubic centimeters of 10 per cent $ZnSO_4 \cdot 7H_2O$ and 3 drops of phenolphthalein solution are added. Approximately N NaOH is slowly added, with constant stirring by means of a thin glass rod with a flattened lower end, until the color is a permanent faint pink. The

volume is brought to the 50-cc. mark. The contents are mixed thoroughly and are then either filtered or centrifuged. Five cubic centimeters of clear fluid and 5 cc. of the copper reagent are *heated 30 minutes in a boiling water bath.* The procedure should be carefully calibrated against known solutions of glucose, galactose, mannose, and glucosamine.

The above procedure has been applied by us (unpublished data of the author and W. D. Sutliff) to the study of the metabolism of the polysaccharide of serum from normal and infected individuals. The polysaccharide, particularly of the serum from persons with infections, is readily metabolized by many bacteria. According to Rimington (1929), it contains mannose and glucosamine. Bierry, Rathery, and Levina (1932), also found galactose. The metabolism of these sugars and glucosamine by the pneumococcus (unpublished data) yields the same products as the metabolism of glucose. Glucosamine therefore should be included in the determination. Many difficulties are encountered in the determination. The long hydrolysis with acid, in the presence of nitrogenous compounds, removes from 1 to 3 per cent of the sugar. The zinc precipitation does not remove all interfering substances; mercury precipitation removes these substances, but it also removes glucosamine. This variable error in the results from analysis of the zinc filtrate is largely compensated by the blank; that is, a sample taken immediately after inoculation which is hydrolyzed and precipitated in exactly the same manner. A further difficulty is the slow rate of oxidation of mannose and galactose by the copper reagent. To obtain the maximum reduction with these sugars, Shaffer recommends a longer period of heating. With 30 minutes of heating with Shaffer's reagent 50, mannose and galactose give practically the same reduction as glucose after 15 minutes of heating (Shaffer and Somogyi, 1933). The reduction of glucose and glucosamine is increased from 1 to 3 per cent.

Lactic acid

The procedure of Friedemann and Graeser (1933) is used. An aliquot of the zinc filtrate from the determination of sugar, or a 2 to 5 cc. sample of the acidified culture, is transferred to a

250 cc. volumetric flask and subjected to $CuSO_4$-$Ca(OH)_2$ precipitation. An aliquot of the filtrate, representing not more than 0.5 cc. of acidified sample is taken for analysis. Since fermented solutions may contain aldehydes or ketones, which analyze as lactic acid, it is advisable to boil the sample for a few minutes before proceeding with the oxidation. Ethyl alcohol does not interfere, nor do any of the non-volatile products, with the exception of 2,3-butylene glycol.[2] The recovery of lactic acid from culture media and the effect of interfering substances have been discussed in previous papers.

Volatile organic acids

A large number of pathogenic bacteria produce only formic and acetic acids (for examples, see table 2). Such cultures are readily analyzed by the author's micro-method (1938), or, if larger quantities of culture medium are available, by the macro-method described below.

Micro-method. Five cubic centimeters of the acidified culture are distilled with steam in the presence of $MgSO_4$. In the case of organisms which produce small quantities of volatile acids, like the staphylococci and streptococci (see table 2), larger samples should be taken. The distillate is acidified and redistilled with tungstic acid-$MgSO_4$. It is aerated with CO_2-free air and titrated with 0.01 N NaOH. The titrated distillate is then redistilled from acid-$MgSO_4$-HgO and again aerated with CO_2-free air and titrated with 0.01 N NaOH. The difference between the two titrations determines formic acid; the second titration determines acetic acid. For details the original paper should be consulted. Before this procedure can be applied to cultures of an organism it must be shown that the acidity of the final distillate is due to only one acid. Behrens (1926), Werkman (1930, 1931a) and Friedemann (1938) have described procedures for identification of acids in a mixture and for their separate quanti-

[2] Before applying the method as described above to the unextracted acidified sample, the analyst should test for the presence of 2,3-butylene glycol. Procedures have been described by Birkenshaw, Charles, and Clutterbuck (1931), and by Brockmann and Werkman (1933).

tative determination. The original papers should be consulted for details.

Macro-method. One hundred cubic centimeters of the acidified culture are transferred to a 500 cc. Kjeldahl flask fitted with a side-neck. Twenty-five grams of $MgSO_4$-$7H_2O$ and 10 cc. of 10 per cent sodium tungstate solution are added. The flask is then connected to the steam generator (a 1 gal. kerosene can) and the distillation is carried out, at first slowly, and until exactly 1 liter of distillate has been collected. Steam enters the solution through a flattened glass bulb of 25 mm. diameter whose lower flat surface rests on the bottom of the flask. A number of small holes are drilled through the flat surface, so that the steam on entering the solution strikes the bottom of the flask. A micro-burner is used to keep the volume at approximately 100 cc. The distillate is mixed and divided into 2 exactly equal portions which are transferred to 1-liter Erlenmeyer flasks. A rapid stream of air is now passed through each for 10 minutes to remove CO_2 and volatile aldehydes and ketones. Five drops of phenolphthalein are added and the titration is carried out with 0.1 N NaOH to a permanent faint pink color. This titration, after correction for recovery of formic acid (see below), determines the total volatile acids. Formic acid is now determined by reduction of $HgCl_2$ by the method of Franzen and Greve (1910) and Fincke (1913). The precipitated HgCl is determined gravimetrically, or iodometrically by the procedure of Utkin-Ljubowzoff (1923). One gram HgCl = 0.979 gram, or 2.12 mM of formic acid. Two cubic centimeters N iodine is equivalent to 1 mM of formic acid.

The recovery of acetic acid is 99 ±0.5 per cent; of formic acid 95 ±1 per cent. The volatility of lactic acid, which is to some extent corrected by the blank, is relatively small with the concentration of $MgSO_4$ (Olmsted and Whitaker, 1928; Olmsted, 1929) recommended in this paper. The salt does not apparently increase decomposition; the same titration values are obtained with or without the salt. Although preliminary deproteinization is desirable, it is not necessary in the majority of cultures. Steam distillation of the acidified culture is simpler and no significant

differences have been noted in the results before and after deproteinization. The technique should be standardized against known quantities of formic acid added to culture medium. The error is rather large, about ±0.5 mM per liter of culture medium.

Volatile alcohols

Before any of the simple, but non-specific, micro-methods widely used in medicolegal work can be applied to cultures of an organism it must be shown that the organism produces only one alcohol. Werkman and Osburn (1931b) and Friedemann (1938) have described relatively simple procedures not only for identification but also for quantitative determination of each of the alcohols in a mixture. If only ethyl alcohol is produced, the method of Friedemann and Klaas (1936) may be used. From 2 to 5 cc. (more if the alcohol content is very small, as in the case of streptococci (see table 2)) of acidified culture are distilled from tungstic acid-$HgSO_4$ and redistilled from $Ca(OH)_2$-HgO. Aliquots of the final distillate are then oxidized by heating 20 minutes in a water bath with alkaline $KMnO_4$. The extent of reduction, determined iodometrically and compared with the reduction in blanks, is a measure of the alcohol. The two distillations remove practically completely acids, amines, aldehydes, ketones, and some of the phenols. The method is thus quite specific for alcohols as a group. The original papers should be consulted for details of technique.

Carbon dioxide

Because foaming is frequently encountered during aeration at the end of the experiment, the culture medium is pipetted into a tall stoppered graduated cylinder. The cylinder is closed after inoculation with a sterile 2-hole rubber stopper provided with two glass tubes, one of which reaches to the bottom. The latter, the inlet tube, is connected with a tower filled with moist soda-lime. The other tube is connected to the liquid absorption tower of Friedemann and Kendall (1929). At the end of the incubation period the inlet tube is disconnected momentarily from the soda-lime tower and the measured volume of N H_2SO_4

is added from the pipette through the inlet tube. Thirty minutes of aeration removes the CO_2, which is absorbed by an excess of standard NaOH solution. The tower is then washed by means of CO_2-free water, an excess of $BaCl_2$ is added, and the free alkali titrated with standard HCl solution. The difference between this titration and that of the blanks determines the CO_2.

Total titratable acidity; undetermined non-volatile acids

Duplicate 25-cc. samples of the acidified culture are pipetted into 300-cc. Erlenmeyer flasks. CO_2 is removed by a rapid stream of CO_2-free air *blown over the surface* for about 10 minutes during which time the flask is frequently gently rotated. Five drops of 1 per cent alcoholic solution of phenolphthalein are added, and the titration is carried out with 0.1 N NaOH to a definite pink color. If the volume of acidified culture available for analysis is small, 5-cc. samples are pipetted into 125-cc. Erlenmeyer flasks and CO_2 is removed as described above. One drop of the indicator solution is added and the titration is carried out with 0.02 N NaOH.

Experiments with lactic, acetic, and formic acids, added to culture media and aerated for 10 minutes as described, gave results which indicated complete recovery. The results are therefore fairly reliable with these three acids in *clear* culture media. However, the end-point in the turbid culture, acidified after 24 hours of growth, is not quite the same as in the blank. The tendency is to over-titrate. This may account for a considerable part of the calculated "undetermined non-volatile" acids shown in column 13 of table 2. It will be noted that in the case of all of the organisms which do not produce "gas," the calculated undetermined acids varied from about 1 to about 2 cc. N acid per liter. The same result was obtained from two of the "gas" producing organisms. This uniformity in results can hardly be due to the culture medium, since two different types of media (see column 3) were used; nor is it likely to be due to systematic errors in the determinations of lactic and the volatile acids, since the yields from the various bacteria varied widely. The uniformity of results from such widely differing types of organisms

indicates over-titration perhaps of the order of 1.5 cc. N acid per liter.

Since the calculation of "undetermined non-volatile acids" is made as follows,

Total titratable acidity − (lactic acid + volatile acids)

it is subject to all of the errors of the methods. Furthermore, basic substances such as NH_3, amines, and amino-acids are always formed; but the yield in carbohydrate-rich media is usually too small to affect seriously the results. This fraction perhaps consists largely of succinic acid, for which at present no satisfactory chemical micro-method is available.

EXPERIMENTAL

Bacteriological technique and sensitivity of analytical methods

A large batch of warmed meat-infusion medium which contained 1 per cent of Witte peptone, 0.5 per cent of phosphate, 2 per cent of serum, and 0.3 per cent of glucose, was inoculated with a virulent Type I pneumococcus. The initial reaction was pH 7.6. This was then transferred to 3 series of flasks. Series A consisted of 13 round-bottom flasks of 50 cc. capacity. B and C consisted of 2 round-bottom flasks of 1 and 2 liter capacities, respectively. All of the flasks were plugged with gauze-cotton and capped with sterile paper. Samples were taken at various times. The initial samples were taken 10 minutes after the flasks had been placed in the water bath. In the case of Series A, the sample consisted of the entire flask to which was added exactly 5 cc. of N H_2SO_4. Five cubic centimeters of culture were withdrawn from flasks B and C at frequent intervals by means of sterile pipettes and transferred to 250-cc. volumetric flasks. The latter contained exactly 5 cc. of N H_2SO_4. Forty cubic centimeters of water were then immediately added; the final concentration of acid therefore was 0.1 N. The numbers of bacteria in the flasks of Series A were determined as recommended by Robertson, Sia and Woo (1924) by plating out dilutions in gelatin-Locke solution of a 1 cc. sample taken just before acidification. The results are shown in table 1 and figure 1.

This experiment illustrates a number of points in technique. First, the method of acidification of samples differed somewhat. Acidification of the entire sample, as in Series A, is safer and is the preferred method. Transfer of many samples, as in Series B and C, in addition to the greater hazard, requires more pipettes, and the culture is more likely to become contaminated. It will

TABLE 1

Experiment 1. Type I pneumococcus (A_5 19R). Anaerobic metabolism

FLASK OR SAMPLE	SERIES	INCUBATION	BACTERIAL COUNT	GLUCOSE USED × 2†	LACTIC ACID
		minutes	*billions per liter*	*mM per liter*	*mM per liter*
1	A	23	0.75	0	0 (4.21)‡
2	A	89	2	0	0 (4.22)
3	A	123	8	0.4	0 (4.23)
4	A	148	30	0.4	0.3 (4.27)
5	A	180*	80	1.0	1.31 (4.43)
6	A	194	100	2.1	1.88
7	A	209	160	2.7	2.87
8	A	225	380	3.8	3.37
9	A	240	450	4.2	4.9
10	A	254	550	7.1	6.3
11	A	270	400	10.0	8.6
12	A	298	680	19.6	14.8
13	A	329	2000	27.9	21.2
13	B	332		29.8	21.6
13	C	333		29.8	21.9

* Definite turbidity due to bacterial growth first noted at this time.

† Multiplication by 2 represents the theoretical lactic acid yield. This expression in terms of lactic acid permits a more ready comparison with the next column. The results in the column are not as accurate as in other experiments because of the high dilution, 1 to 50 with an initial sugar content of 300 mgm. per cent; the dilution should have been 1 to 10.

‡ The final titration average with 0.005 N iodine (sample of 0.4 cc.) is shown in parenthesis.

be noted that each of the three series of results for lactic acid shown in figure 1 increases in a regular manner, and all agree, within the limits of experimental error, with the curve drawn through the data. The consistency of the results demonstrates the efficacy of the acid to stop metabolic activities instantly in the rapidly growing culture, even when the acid is added to the

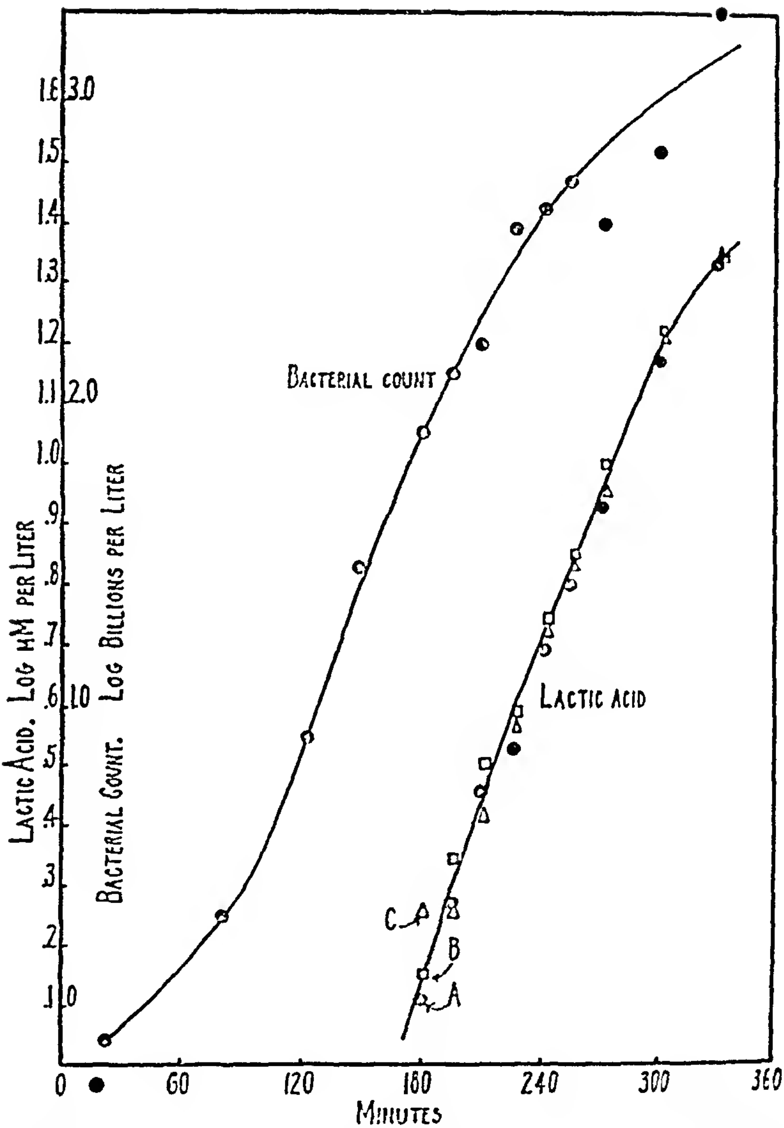

FIG. 1. RATE OF METABOLISM AND POPULATION INCREASE OF PNEUMOCOCCUS, TYPE I (STRAIN A_1) IN A 0.3 PER CENT GLUCOSE, BEEF-INFUSION PEPTONE MEDIUM AT 37.5°C.

Three sets of data are shown for lactic acid: *A*, data from 13 small flasks, each containing 50 cc. of culture medium, the contents acidified at the time indicated; *B*, 5 cc. samples, taken from 1000 cc. of culture medium in a 1 liter flask; *C*, 5 cc. samples taken from 2000 cc. of culture in a 2 liter flask and analyzed as in *B*. See also table 1.

whole culture, as in Series A. Secondly, the growth was carried out in flasks of different size. In the past, in order to obtain complete metabolic data, it has been necessary to grow the organism in a large volume of culture medium. With the newer, more sensitive, methods it is possible to make the same analyses on smaller volumes—if necessary, 25 cc. This experiment shows that the results are the same irrespective of the volume, provided the culture medium and the conditions throughout are exactly the same. Finally, metabolic data were obtained from small volumes contained in a *series* of flasks. In order to identify minute quantities of metabolic products, it may be necessary to work over a large volume of culture medium. Again, it may be desired to obtain complete analytical data at various stages in the fermentation. For these purposes growth of the organism in many flasks or bottles, rather than in a single flask, is recommended. The smaller containers are more easily handled and, because of the relatively thicker walls, the possibility of breakage is minimized.

This experiment demonstrates also the degree of sensitivity of some of the described micro-methods. This can be seen by comparing the data for glucose and lactic acid, shown in table 1, with the bacterial count. Titration data for the lactic acid determination in the first 5 samples are shown in parenthesis in the last column. This culture medium initially contained lactic acid. In order to indicate any increase of lactic acid in the culture the titration must be at least 0.1 cc. greater than the preceding samples, since the permissible error in titration is ±0.05 cc. (±1 drop) of 0.005 N iodine solution. The methods for glucose and ethyl alcohol are more sensitive; the methods for volatile acids are slightly less sensitive.[4] It will be noted that the analyses indicated no significant increase of lactic acid beyond the experimental error, or decrease of glucose (see footnote 2, table 1), until the number of pneumococci in this experiment had increased to about 80 billion per 1. The writer has obtained

[4] The sensitivity of these methods is of about the same order of magnitude as those used by Bayne-Jones (1929) and Bayne-Jones and Rhees (1929) and Walker, Winslow, Huntington and Mooney (1934).

results of about the same order with other bacteria. At this point a faint turbidity is first noted and the bacterial counts, which up to this point have been small, begin to increase with great rapidity. Within the next 3 to 5 hours, with optimum conditions, fermentation by the pneumococcus is practically completed. When plotted logarithmically, as in figure 1, the rate of increase of population is approximately the same during the first 3 hours as in the later phase. In the experiment shown in figure 1, the number of preceding generations, thus, is about the same as the number of generations in the subsequent period for which the analytical methods apply. *With the present methods (where the inoculum is small) it is therefore impossible to obtain reliable metabolic data from the young culture representing the first third or half of the rapid growth phase.*

Reliability of analytical methods

Two types of media were used. Medium A is the same as was used in the rate experiment described above. Five different batches, made at various times from 1933 to 1937, are designated in table 2 by numbers A1 to A5. All contained 1 per cent of added glucose. Medium B consisted of 0.3 per cent of meat extract (Libby), 1 per cent of Difco Bacto-peptone, 0.75 per cent of Na_2HPO_4, and 1 per cent of glucose. The initial reaction was pH 7.6. Since comparative analyses were to be made by many methods, exactly 300 cc. were pipetted into tall 12-ounce dispensing bottles. The pneumococci were the same as used by Robertson, Coggeshall and Terrell (1933) in previous studies. The colonies on whole-blood agar consisted entirely of the smooth variety. A_5 is highly virulent for mice and rabbits; A_3 is somewhat less virulent, and A_4 is relatively avirulent. The organisms inoculated into bottles 6 to 15 inclusive have been in the writer's collection for a number of years. Their virulence was not tested. With the exception of the last 3 organisms (see table 2), the staphylococci and streptococci were isolated within the week preceding the experiment either from a lesion or from a blood culture. The last 3 were organisms of tested virulence obtained from Dr. George F. Dick. The identity of the organisms was

TABLE 2

Metabolic products of pathogenic bacteria in sugar-rich media after 24 hours at 37.5°C. Results are expressed as millimols or cubic centimeters N acid per liter

NUMBER	ORGANISM	CULTURE MEDIA*	GLUCOSE × 2†	LACTIC ACID	ALCOHOL: Oxidation to fatty acids: K	ALCOHOL: Oxidation to fatty acids: Calculated alcohol	ALCOHOL: Direct determination by Friedemann and Klaas method	VOLATILE ACIDS: Macro-method: Formic acid	VOLATILE ACIDS: Macro-method: Acetic acid	VOLATILE ACIDS: Micro-method: K	VOLATILE ACIDS: Micro-method: Acetic acid	UNDETERMINED: Non-volatile acids	UNDETERMINED: Carbon (C_3 results in col. 4 = 100 per cent)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
			mM C_3	mM		mM	mM	mM	cc.N		cc.N	cc.N	per cent
1	Pneumococcus, I (A_5)	A1	44.3	36.7			1.6	2.3	1.0			2.4	14‡
2	Pneumococcus, II (A_4)	A1	39.9	28.8			3.3	5.8	2.8			2.2	13‡
3	Pneumococcus, II (A_4)	A6	67.1	25.3	86.2	12.2		25.2	11.7	85.6	12.7	2.1	13
4	Pneumococcus, III (A_3)	A1	41.9	32.2			2.4	4.7	2.3			2.2	12‡
5	Pneumococcus, III (A_3)	A5	59.7	43.2	87.6	4.6		10.3	5.1	85.9	4.5	2.7	11
6	*Vibrio cholerae* 59	B1	58.5	11.6	86.5	16.3	16.8	29.9	12.3	87.4	13.2	0.6	30
7	*Shigella dysenteriae* Flexner 4	B1	65.3	28.6	86.6	13.9	13.8	27.2	14.1	88.3	14.3	2.8	14
8	*Eberthella typhosa* 41	B1	61.2	17.1	85.7	17.9		36.6	17.3	87.6	17.2	1.7	14
9	*Salmonella suipestifer* 58	B1	65.8	31.8	87.0	14.3	14.8	13.3	13.5	87.6	14.0	2.3	16
10	*Salmonella typhimurium* 33	B1	79.9	31.8	86.5	20.2	20.4	17.4	15.6			8.3	23
11	*Salmonella enteritidis* 10	B1	80.9	40.5	86.8	16.7	17.0	11.2	13.6	88.6	12.8	7.7	21
12	Friedländer's bacillus 57	B1	82.3	37.2	86.0	18.9		9.8	16.4	87.9	16.2	2.1	22
13	*Escherichia coli* 14	B1	90.5	36.9	85.9	20.6		8.9	18.7	87.6	18.7	7.7	27
14	*Salmonella schottmuelleri* 29	B1	96.8	42.5	87.1	21.3	21.9	3.8	16.9	87.1	17.3	8.1	28
15	*Escherichia acidilactici* 12	B1	101.8	46.5	86.0	23.6	24.4	0.6	17.1	87.1	16.6	14.2	28
16	*Staphylococcus aureus* (hemolytic)	A2	64.6	61.7	87.1	7.4				91.7	0.5		
17	*Staphylococcus aureus* (hemolytic)	A2	69.9	51.0	86.1	4.2					−0.3		
18	*Staphylococcus aureus* (hemolytic)	A2	61.2	50.8	84.5	6.6					0.2		
19	*Staphylococcus aureus* (hemolytic from puerperal fever)	A3	60.3	51.3			6.3	1.8	0.6			1.8	8
20	*Staphylococcus aureus* (non-hemolytic)	A3	60.7	50.6			7.2	2.4	0			1.2	9
21	*Staphylococcus aureus* (non-hemolytic)	A3	64.2	49.3			10.9	3.5	1.7			2.8	8
22	*Staphylococcus albus*	A3	36.6	31.1			3.5	0.2	1.4			−1.5	6
23	*Streptococcus hemolyticus*	A2	49.6	45.3	84.5	0.3				84.9	0.7		
24	*Streptococcus hemolyticus*	A2	48.9	44.0	87.8	0.4				85.5	1.2		
25	*Streptococcus hemolyticus* (puerperal fever)	A3	33.9	31.7			0.3	0.6	1.6			1.9	2
26	*Streptococcus hemolyticus*	A3	41.0	39.7			0.2	0.7	1.8			0.2	0
27	*Streptococcus hemolyticus*	A3	27.6	27.9			0.2	1.1	1.1			−.6	+5
28	*Streptococcus scarlatinae*	A3	61.8	54.5			2.7	2.8	2.3			1.0	5
29	*Streptococcus scarlatinae*	A3	55.5	47.7			0.7	2.0	1.9			1.1	10
30	*Streptococcus erysipelatis*	A4	43.1	38.4			1.1	1.4	2.4			0.7	5

* The media were inoculated on the following dates: A1, May 8, 1933; A2, June 8, 1937; A3, February 27, 1935; A4, April 1, 1935; A5, February 10, 1937; B1, November 4, 1936.

† See footnote, table 1.

‡ The increase of CO_2 in pneumococcus cultures 1, 2, and 4 was 0.07, 0.04, and 0.06 mM, respectively.

checked by growth on various sugars. Their purity was determined by smear and subculture immediately preceding the inoculation and just before termination of growth by acid. The bottles were incubated for 24 hours in the water bath at 37.5°C. The results, expressed as millimols (mM) or cubic centimeters N acid per liter,[5] are shown in table 2.

The results of determination of alcohol by two methods are shown in columns 7 and 8. Every organism produced ethyl alcohol; the distribution constant of the fatty acid obtained by oxidation with chromic acid (see column 6) in every instance was identical with that of acetic acid. The method of Friedemann and Klaas therefore can be applied to these organisms. The results in column 8, obtained by this procedure, agree within the limits of experimental error (±2 per cent) with those shown *in column 7, obtained by the Friedemann (1938) method.*

The fatty acids were determined by 2 methods: the micromethod of Friedemann (1938), which also determines the distribution constant, and the macro-method described in this paper. Every organism produced acetic acid; the distribution constant, K, of the acid not removed by HgO was identical with that of acetic acid. The results in columns 10 and 12 agree within the limits of error of each of the methods.

The consistency of the data as a whole should be noted. These results were obtained from widely differing types of bacteria grown in different media at various times. The sugar consumption and the relative yields of products differed greatly. Yet it is interesting to find certain striking similarities. Thus, the ratio of ethyl alcohol to formic and acetic acids was the same in pneumococcus cultures, approximately 1 to 2 to 1, in both media A1 and A2. Similar maximum ratios were also observed in cultures 6, 7, and 8. The significance of the low minimum value for "undetermined non-volatile acids," approximately 1.5 cc. N acid,

[5] This method of expressing results has many advantages. As an example, the results for alcohol, formic and acetic acids from the pneumococcus suggest a causal relation, while no such relation can be seen in the results from staphylococci. The underlying chemical reactions can thus be more readily detected. Furthermore, the osmotic relations of the constituents, the percentage yields and other calculations can be more readily made. See footnote of table 1.

has already been discussed. It is the same, for example, in all of the pneumococcus cultures, even though the first set of analyses was made 4 years earlier. It is the same in culture 2, which contained more of lactic acid and considerably less of volatile acids than culture 3. Finally, not only the small percentage but also the uniformity of the results for the undetermined carbon should be noted in the case of organisms which produce principally lactic and only small quantities of volatile acids. The calculated results are shown in column 14. In these calculations, lactic acid, alcohol, and the volatile acids are assumed to be derived solely from the metabolized sugar. The yield of these products, as determined by the methods described in this paper, accounts for 90 to 95 per cent of the sugar used by staphylococci and streptococci. The metabolic processes of even such simple cells as bacteria are extremely complex. It is reasonable to assume that some of the carbohydrate was used to build the cell structure. Some may have yielded glycerol or succinic acid; some may have been oxidized to CO_2. Other products, besides those analyzed, are therefore to be expected.

SUMMARY AND CONCLUSIONS

A procedure is outlined for safely conducting metabolic experiments with pathogenic microörganisms. Accurately measured volumes of the medium are pipetted into small containers, preferably bottles, and inoculated with a measured quantity of a culture or suspension of the organism. The culture is then incubated, preferably in a water bath, and acidified at any desired time to stop growth and metabolic activities. The acidified culture is tightly stoppered and kept in the refrigerator until ready for analysis.

Analytical procedures are described in detail for reducing substances (glucose), hydrolyzable sugar, alcohols, volatile acids, lactic acid, carbon dioxide and total titratable acids. These were applied to carbohydrate-rich meat infusion or meat extract media which contained from 0.3 to 0.75 per cent of phosphate buffer, and which were enriched with 1 per cent of peptone. Of the alcohols, only ethyl alcohol was produced. The volatile acids

consisted of formic and acetic acids only. Metabolic data are presented which show that lactic acid, alcohol, and volatile acids account for 90 to 95 per cent of the sugar metabolized by staphylococci and streptococci. These three products accounted for from 85 to 90 per cent of the metabolized sugar in 5 cultures of the pneumococcus and 1 culture each of *Shigella dysenteriae* Flexner, *Eberthella typhosa*, and *Salmonella suipestifer*. About 75 per cent was similarly accounted for in single cultures of *Escherichia coli*, Friedländer's bacillus, *Salmonella typhi-murium*, *Salmonella enteritidis*, *Salmonella schottmülleri*, *Escherichia acidi-lactici* and *Vibrio cholerae*. The yield of metabolic products is great enough so that a complete analysis by all of the methods described can be made on a minimum of 25 cc. of the culture.

The metabolic data demonstrate the effectiveness of acid, both for stopping metabolic activities and for preserving the culture. Consistency, as a whole, of the data from bacteria differing widely in their cultural and morphological characters is evidence of the reliability of the analytical procedures. Where the inoculum is small, with the present methods it is impossible to obtain reliable complete metabolic data from the young culture representing the first third or half of the rapid growth phase.

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REFERENCES

Bayne-Jones, S. 1929 Bacterial calorimetry. I. General considerations. Description of differential microcalorimeter. Jour. Bact., 17, 105-122.

Bayne-Jones, S., and Rhees, H. S. 1929 Bacterial calorimetry. II. Relationship of heat production to phases of growth of bacteria. Jour. Bact., 17, 123-140.

Behrens, W. U. 1926 Quantitative Analyse von Gemische flüchtiger Fettsäuren durch Verteilung zwischen Äthyläther und Wasser. Zt. anal. Chem., 69, 97-107.

Bierry, H., Rathery, F., and Levina, Mlle. 1932 Le sucre protéidique. Paris Médical, 83, 137-144.

Birkinshaw, J. H., Charles, J. H. V., and Clutterbuck, P. W. 1931 Quantitative examination by the carbon balance sheet method of the types of products formed from glucose by species of bacteria. Biochem. Jour., 25, 1522-39.

BROCKMANN, M. C., AND WERKMAN, C. H. 1933 Determination of 2,3-butylene glycol in fermentations. Ind. Eng. Chem., Anal. Ed., 5, 206–207.

COHEN, B. 1922 The effects of temperature and hydrogen ion concentration upon the viability of *Bact. coli* and *Bact. typhosum* in water. Jour. Bact., 7, 183–230.

FINCKE, H. 1913 Nachweis und Bestimmung der Ameisensäure. Biochem. Zt., 51, 253–287.

FRANZEN, H., AND GREVE, G. 1910 Uber die Vergärung der Ameisensäure durch *Bacillus prodigiosus*. Zt. physiol. Chem., 64, 169–261.

FRIEDEMANN, T. E. 1928 On the determination of lactic acid in sugar solutions decomposed by alkali. Jour. Biol. Chem., 76, 75–87.

FRIEDEMANN, T. E. 1938 The identification and quantitative determination of volatile alcohols and acids. Jour. Biol. Chem. (To be published soon.)

FRIEDEMANN, T. E., COTONIO, M., AND SHAFFER, P. A. 1927 The determination of lactic acid. Jour. Biol. Chem., 73, 335–358.

FRIEDEMANN, T. E., AND GRAESER, J. B. 1933 The determination of lactic acid. Jour. Biol. Chem., 100, 291–308.

FRIEDEMANN, T. E., AND KENDALL, A. I. 1929 The determination of carbon and carbon dioxide. Jour. Biol. Chem., 82, 45–55.

FRIEDEMANN, T. E., AND KLAAS, R. 1936 The determination of ethyl alcohol. Jour. Biol. Chem., 115, 47–61.

HARDEN, A. 1901 The chemical action of Bacillus coli communis and similar organisms on carbohydrates and allied compounds. Jour. Chem. Soc., 79, 610–628.

HEWITT, L. F. 1932a Lactic acid production by haemolytic streptococci. Biochem. Jour., 26, 208–217.

HEWITT, L. F. 1932b Glucose breakdown by pneumococcus variants and the effect of phosphate thereon. Biochem. Jour., 26, 464–471.

MAGEE, M. C., AND SMITH, H. G. 1930 A study of methods for the estimation of reducing sugar in bacteriological media. Jour. Bact., 19, 125–132.

OLMSTED, W. H., AND WHITAKER, W. M. 1926–27 Steam distillation of volatile fatty acids from a saturated salt solution. Proc. Soc. Exp. Biol. and Med., 24, 897–98.

OLMSTED, W. H., WHITAKER, W. M., AND DUDEN, C. W. 1929–30 Steam distillation of the lower volatile fatty acids from a saturated salt solution. Jour. Biol. Chem., 85, 109–114.

RIMINGTON, C. 1929 The isolation of a carbohydrate derivative from serum-proteins. Biochem. Jour., 23, 430–443.

ROBERTSON, O. H., SIA, R. H. P., AND WOO, S. T. 1924 The protective action of gelatin for pneumococci in suspension. Jour. Exp. Med., 39, 199–218.

ROBERTSON, O. H., COGGESHALL, L. T., AND TERRELL, E. E. 1933 Experimental pneumococcus lobar pneumonia in the dog. III. Pathogenesis. Jour. Clin. Invest., 12, 467–493.

SHAFFER, P. A., AND HARTMANN, A. F. 1920–21 Methods for the determination of reducing sugars in blood, urine, milk, and other solutions. Jour. Biol. Chem., 45, 365–390.

SHAFFER, P. A., AND SOMOGYI, M. 1933 Copper-iodometric reagents for sugar determination. Jour. Biol. Chem., 100, 695–713.

SOMOGYI, M. 1930 A method for the preparation of blood filtrates for the determination of sugar. Jour. Biol. Chem., 86, 655-663.

STILES, H. R., PETERSON, W. H., AND FRED, E. B. 1926 A rapid method for the determination of sugar in bacterial culture. Jour. Bact., 12, 427-430.

UTKIN-LJUBOWZOFF, L. 1923 Eine neue titrimetrische Bestimmungsmethode der Ameisensäure. Biochem. Zt., 137, 205-208.

WALKER, H. H., WINSLOW, C.-E. A., HUNTINGTON, E., AND MOONEY, M. G. 1934 The physiological youth of a bacterial culture as evidenced by cell metabolism. Jour. Bact., 27, 303-323.

WERKMAN, C. H. 1930 Determination of fatty acids in mixtures by partition between isopropyl ether and water. Ind. Eng. chem., Anal. Ed., 2, 302-304.

WERKMAN, C. H. 1931a Application of partition method to quantitative determination of acetic, propionic, and butyric acids in mixture. Ind. Eng. Chem., Anal. Ed., 3, 264-265.

WERKMAN, C. H., AND OSBURN, O. L. 1931b Determination of butyl and ethyl alcohols in mixtures. Ind. Eng. Chem., Anal. Ed., 3, 387-392.

WINSLOW, C.-E. A., AND LOCKRIDGE, E. E. 1906 The toxic effect of certain acids upon typhoid and colon bacilli in relation to the degree of their dissociation. Jour. Infect. Dis., 3, 547-571.

FACTORS INFLUENCING THE RATE OF FERMENTATION OF STREPTOCOCCUS LACTIS

OTTO RAHN, C. P. HEGARTY AND R. E. DEUEL

Laboratory of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca, N. Y.

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The rate of lactic fermentation can be measured readily with centrifuged cells in glucose solution. Virtanen and associates have done this with cells of *Streptococcus lactis* and *Lactobacillus casei*, mostly with dried or toluene-treated cells, but one series (1927) deals with living cells. Their results in regard to the optimal pH differ widely from ours. Several other factors are reported here which Virtanen and his associates did not study.

METHOD

Our studies are limited to strains 14 (isolated from plants) and 125 (from milk) of *S. lactis*. Virtanen grew his bacteria in whey, i.e., in a lactose medium. We used a broth made of 5 grams peptone, 5 grams tryptone, 10 grams glucose, 36.6 grams $Na_2HPO_4 \cdot 12H_2O$ and 4.2 grams KH_2PO_4 per liter. This same medium plus 15 grams agar was used for plate counts. Inoculation was heavy, 5 to 10 cc. of a young culture per liter. The cells were centrifuged when the culture was 12 to 14 hours old. The sedimented cells were re-suspended in a phosphate buffer of the same concentration as given above. This suspension was added to the test medium in such quantity that the cells from 250 cc. of culture were condensed in 50 cc. of the test medium. Thus, we hoped to avoid growth.

The test medium consisted of the same 2 per cent buffer (36.6 grams $Na_2HPO_4 \cdot 12H_2O$ = 15.8 grams anhydrous, plus 4.2 grams KH_2PO_4) to which 3 or 5 grams peptone and 20 grams glucose per liter were added. The peptone is essential. All cultivation and all fermentation tests were carried out at 30°C.

We measured the lactic acid by titration of 5 cc. of the cell suspension with N/10 NaOH and phenolphthalein. Virtanen states that this method is not accurate when the phosphate concentration is 2 per cent or higher. By using a color standard for titration, the determinations were fairly accurate even with 8 per cent phosphates, although the endpoint becomes less sharp as the phosphate concentration increases. All experiments except the first one were made with a 2 per cent buffer. Since titration permitted 8 to 10 determinations in half-hour intervals, it had great advantages over one or two determinations after 2 to 8 hours. The curve of acid formation was quite useful in the interpretation of the results.

The basis of comparison was always the fermenting capacity, i.e., the milligram lactic acid produced per cell per hour. This was computed from titration and plate counts. Usually, the bacteria did not multiply during the experiment, and the fermenting capacity remained constant until enough acid had been formed to change the pH.

EFFECT OF pH AND OF BUFFER CONCENTRATION

An extensive experiment is most apt to show what kind of results could be obtained by our method. In this set of data, an acetate phosphate buffer was used since phosphate alone did not buffer sufficiently at pH 4.5. The mineral concentration was so arranged that it was very nearly 8 per cent throughout the entire pH range. Of these buffers, dilutions were made to contain 6, 4 and 2 per cent of buffering substances. All buffers contained 2 per cent glucose and 0.3 per cent peptone. The bacteria were obtained by means of a super-centrifuge and their concentration in the final experiments was 4.0×10^9 cells per cubic centimeter.

Table 1 shows the acid produced, and table 2 the fermenting capacities computed from the periods of constant rates. Slight irregularities are unavoidable in these strongly buffered solutions. Fermentation was most rapid at pH 7.0, and almost as rapid at pH 7.5 and 8.0. Even at pH 9.0, the rate was faster than at pH 6.0. This result differs greatly from Virtanen's who found

TABLE 1

Effect of pH and of buffer concentration upon the rate of fermentation of Streptococcus lactis 125

pH	PER CENT OF LACTIC ACID FORMED AFTER									pH AFTER	
	0.5 hour	1.0 hour	1.5 hours	2.0 hours	2.5 hours	3.0 hours	4.0 hours	5.0 hours	6.0 hours	4 hours	6 hours
In 2 per cent buffer concentration											
4.5	0.022	0.047	0.050	0.074	0.085	0.085	0.090	0.095		4.3	4.26
5.0	0.036	0.072	0.108	0.135	0.153	0.162	0.216	0.216	0.243	4.62	4.65
6.0	0.054	0.126	0.180	0.216	0.253	0.280	0.342	0.360	0.378	4.65	4.80
7.0	0.072	0.144	0.198	0.262	0.315	0.360	0.405	0.450		5.34	
7.5	0.072	0.144	0.207	0.280	0.350	0.414	0.513	0.530	0.585	5.66	5.28
8.0	0.072	0.135	0.207	0.270	0.342	0.396	0.477	0.522		5.80	5.28
9.0	0.072	0.126	0.216	0.288	0.342	0.414	0.495	0.558	0.602	5.70	5.44
In 4 per cent buffer concentration											
4.5	0.018	0.018	0.032	0.045	0.054	0.054	0.054	0.054	0.072	4.45	4.48
5.0	0.018	0.054	0.090	0.099	0.081	0.172	0.172	0.180	0.190	4.77	5.00
6.0	0.018	0.126	0.162	0.216	0.270	0.315	0.342	0.424	0.486	5.16	5.16
7.0	0.126	0.216	0.306	0.396	0.450	0.540	0.675		0.722	5.9	
7.5	0.090	0.171	0.254	0.334	0.414	0.506	0.640	0.730	0.745	6.32	5.85
8.0	0.063	0.135	0.207	0.342	0.433	0.522	0.666			6.5	6.02
9.0	0.063	0.126	0.198	0.270	0.360	0.450	0.576	0.702	0.722	6.7	6.11
In 6 per cent buffer concentration											
4.5	0.014	0.018	0.040	0.090	0.090	0.090	0.090	0.090	0.072	4.45	4.55
5.0	0.009	0.036	0.090	0.108	0.081	0.099	0.099	0.108	0.134	4.82	5.05
6.0	0.018	0.144	0.234	0.243	0.306	0.360	0.426	0.504	0.575	5.40	5.50
7.0	0.054	0.126	0.288	0.350	0.450	0.531	0.655	0.755	0.827	6.22	
7.5	0.081	0.144	0.216	0.300	0.423	0.476	0.621	0.746	0.818	6.8	6.64
8.0	0.090	0.153	0.234	0.306	0.414	0.495	0.630	0.772	0.910	6.8	6.63
9.0	0.036	0.108	0.198	0.234	0.306	0.360	0.495	0.639	0.700	7.0	6.72
In 8 per cent buffer concentration											
4.5	0.009	0.070	0.110	0.110	0.110	0.110	0.110		0.110	4.52	4.50
5.0	0.054	0.090	0.126	0.144	0.099	0.099	0.144	0.090	0.153	4.90	5.00
6.0	0.072	0.072	0.144	0.207	0.243	0.306	0.396	0.441	0.512	5.54	5.65
7.0	0.126	0.144	0.234	0.288	0.396	0.476	0.592	0.655	0.772	6.45	
7.5	0.036	0.099	0.144	0.225	0.234	0.387	0.531	0.621	0.729	6.77	6.47
8.0	0.063	0.090	0.153	0.207	0.261	0.288	0.396	0.512	0.549	7.13	6.95
9.0	0.009	0.081	0.099	0.117	0.153	0.189	0.225	0.288	0.306	7.55	7.50

the rate to be highest at pH 6.2, and 40 to 50 per cent lower at pH 7.8. His test temperature was 20°. Probably, this and the difference in strains accounts for the difference in results.

In the buffer solutions of pH 8 and 9, acid formation tends to reduce the pH and to bring it nearer to the optimum of 7. The concentrated buffers will prevent this by their strong buffering effect, and therefore retard the rate of fermentation. The opposite is true at pH 4.5 and 5. Acid production decreases the pH and removes it still further from the optimal range. The more concentrated buffers prevent this further drop, and therefore show the higher rate of fermentation. For pH 7 to 8, the optimal buffer concentration is 4 per cent.

That the rate is constant for a longer time in the concentrated solutions, need hardly be mentioned. In the 8 per cent buffer solutions, however, the fermentation mechanism seems to be damaged to quite an extent because the rate falls off quickly, and the final amount of acid produced is decidedly lower than in the 6 per cent buffers at any pH except the two lowest.

TABLE 2

Rates of fermentation from table 1 expressed in 10^{-11} mgm. lactic acid per cell per hour

pH	BUFFER CONCENTRATION			
	2 per cent	4 per cent	6 per cent	8 per cent
4.5	0.8	0.5	1.1	1.3
5.0	1.7	1.2	1.5	1.8
6 0	2.7	2.7	3.0	2.6
7 0	3.3	5.0	4.3	4 0
7 5	3.5	4 2	4.0	3.2
8.0	3.3	4.4	4.1	2 6
9.0	3.5	3 8	3.0	1.6

EFFECT OF CELL CONCENTRATION

If we are dealing here with the zymase alone, i.e., uninfluenced by other cell functions, the rate must be strictly proportional to the cell number. Several experiments were made to test this.

Strain 125

cells per cc.	*mgm. per cell per hour*
3,600,000,000	6.0×10^{-11}
1,800,000,000	6.4×10^{-11}
900,000,000	8.0×10^{-11}

Strain 14

cells per cc.	*mgm. at 30°*	*mgm. at 45°*
1,000,000,000	23.2×10^{-10}	35.5×10^{-10}
500,000,000	28.8	34.6
250,000,000	32.4	38.4
125,000,000	37.8	40.4
62,500,000	50.4	46.0

A parallel experiment with the strain 14 was made where the cells were killed during washing by a too high temperature of the centrifuge. Fermentation was weaker, but measurable. The cell ratio was 8:4:2:1 while the acid formed showed the ratio 7.85:4:2.00:1.28.

The experiments with living cells show an increasing rate of fermentation when fewer cells are present. This is due to multiplication of the cells if the concentration is less than 10^9 per cubic centimeter. At 45° where multiplication becomes extremely slow, the increase in rate of fermentation by the lower cell concentrations is comparatively small.

EFFECT OF GLUCOSE CONCENTRATION

Slator (1906) has shown that alcoholic fermentation by yeast depends upon the sugar concentration only if less than 1 per cent is present. Above 1 per cent, the rate is practically independent of the sugar concentration. Our method made the study of this question quite simple, and the result is shown in table 3. The data are averages of 10 complete experiments made by 10 different workers with the same bacterial suspension in 2 per cent buffer solution of pH 6.4, with varying amounts of glucose. For some unknown reason, the fermentation did not start until about 5 or 10 minutes after the cells were placed in contact with the glucose, and therefore, the rate for the first half hour is low throughout.

The buffer concentration was 2 per cent, and the pH remained constant at 6.4 until about 0.35 per cent of lactic acid had been formed. This occurred between 1.5 and 2 hours at the higher glucose concentrations. After that, it decreased rapidly, and, with it, decreased the rate of fermentation.

The amount of sugar available at any time can be roughly estimated by subtracting the total acid formed from the original glucose concentration. Thus, after 0.5 and 1 hour, the glucose of the second flask had decreased from 0.25 to 0.15 and 0.07 per cent respectively.

The lower half of the table shows the rate of fermentation to be uniform for the second and third half-hour period, from 0.5

TABLE 3

Effect of the concentration of glucose

TIME	GLUCOSE CONCENTRATION					
	0.125 per cent	0.25 per cent	0.50 per cent	1.00 per cent	2.00 per cent	4.00 per cent
	Total lactic acid formed					
hours						
0.5	0.073	0.100	0.090	0.084	0.084	0.090
1.0	0.091	0.179	0.202	0.204	0.206	0.206
1.5	0.093	0.207	0.321	0.333	0.326	0.342
2.0	0.093	0.210	0.354	0.415	0.394	0.380
2.5	0.093	0.210	0.358	0.469	0.459	0.428
3.0	0.093	0.210	0.360	0.489	0.480	0.464
3.5	0.093	0.210	0.361	0.502	0.489	0.478
Final pH	6.4	6.4	6.1	4.4	4.4	4.4
	Per cent lactic acid formed per half hour					
half hours						
1st	0.073	0.100	0.090	0.084	0.084	0.090
2nd	0.018	0.079	0.113	0.120	0.122	0.116
3rd	0.002	0.027	0.118	0.129	0.120	0.136
4th	0	0.003	0.034	0.083	0.068	0.038
5th	0	0	0.004	0.054	0.065	0.048
6th	0	0	0.002	0.020	0.022	0.036
7th	0	0	0.001	0.013	0.008	0.014

to 4 per cent sugar. With 0.125 per cent sugar, the initial rate was fairly high, and with 0.25 per cent, almost as high as the highest during the first half hour, but in both cases, the glucose concentration during the second half hour fell below 0.1 per cent. With 0.5 per cent initial sugar, fermentation was constant for the first 1.5 hours (0.18 per cent glucose remaining), but dropped decidedly during the next half-hour. It seems safe to state from these results that the rate of lactic fermentation is independent

of the glucose concentration when it amounts to at least 0.2 per cent of the culture medium, provided, of course, that the pH does not decrease.

EFFECT OF Na-LACTATE

Two experiments with strain 14 showed very little effect. Even the highest concentrations corresponding to 3.2 per cent lactic acid still permitted very noticeable fermentation though the rate had been reduced to about one-third. Rogers and Whittier (1928) had found in milk 0.1829 molar acid (= 2.2 per cent Na-lactate) to be completely inhibiting; but in an artificial medium with 0.5 K_2HPO_4, no constant relation could be found.

TABLE 4

EXPERIMENT I		EXPERIMENT II	
Na-lactate	Fermenting capacity	Na-lactate	Fermenting capacity
per cent	*10^{-10} mgm. per cell per hour*	*per cent*	*10^{-10} mgm. per cell per hour*
0	22.9	0	25.6
0.2	22.9	1	19.2
0.4	21.9	2	18.7
0.8	20.4	3	12.9
		4	9.6

This latter experiment corresponds more closely to ours than the one with milk. It must be expected that our strongly buffered test medium would give results different from those obtained in milk or whey.

EFFECT OF AERATION

Air, oxygen and nitrogen were bubbled through cell suspensions of strain 125 in wide test tubes as rapidly as this could be done without losing some of the culture by foaming. The effect in a preliminary test was slight, 19 per cent retardation by oxygen and 17 per cent by air. In the final experiment, the fermenting capacities were

Quiet controls		8.65×10^{-10} mgm.	
Agitated with current of	air	6.7	= 22 per cent reduction
	oxygen	4.8	= 44 per cent reduction
	nitrogen	10.3	= 20 per cent increase
In vacuum		8.60	

The number of viable cells was not changed materially during the 4.5 hours of the experiment. We must conclude that the enzyme action as such is in some way inhibited by too much oxygen. The great reducing power of streptococci as shown by their effect upon litmus and methylene blue seems to be sufficient to eliminate the harmful effect of oxygen in a quiet suspension. Oxygen is removed more rapidly than it can diffuse into the culture, and therefore, no difference was observed between vacuum and regular control. However, the increase by nitrogen does not fit into this picture.

AGE OF CELLS

In the most extensive of our experiments, the age varied from 2 to 48 hours. By age is meant the time from inoculation until the beginning of centrifugation. For centrifugation and re-suspension, 35 more minutes were required before the first titration could be made. In order to avoid lag, the inoculum always consisted of young cells. Thus, to obtain the 2-hour-old cells, 1,500 cc. were inoculated with 60 cc. of a 4-hour-old culture which had been obtained from a 9-hour culture. The cells from 250 cc. of culture were re-suspended in 50 cc. of the test medium, with the exception of the 2- and 4-hour-old cultures. We expected rather small numbers of cells, and hence, the cells from 500 cc. of the 4-hour culture, and from 1500 cc. of the 2-hour culture were concentrated in 50 cc. of the test medium.

Of each suspension, a plate count was made at once, and again after 4 hours when the titrations were finished. No increase in cells beyond the limits of error was observed in any case.

Acid formation proceeded in a very regular way, the only abnormal set being the two 8-hour-old cultures which had an initial delay, and later showed further inconsistencies. The fermenting capacities showed a surprising uniformity.

Age of cells in hours	2	4	6	8	10	12	14	16	18	24	36	48
10^{-11} mgm. per cell per hour	7.4	6.4	8.8	[6.0]	8.2	6.8	5.5	5.2	6.3	6.3	6.8	[illegible]

The fermenting capacity is surprisingly uniform. The very short but very greatly increased rate of metabolism observed by

Bayne-Jones and Rhees (1929) and by Walker, Winslow, Huntington and Mooney (1934) was not evident. These authors observed that cells just coming out of lag, before the plate count showed a doubling of the cells, had a rate of metabolism 4 to 8 times as high as a few hours later, during the logarithmic phase. While our cells had not become old, on account of frequent transfers, they had not recently come out of lag. To induce this condition, 1.5 liters of a 36-hour-old culture were transferred to 13.5 liters of fresh medium. Every hour, part of this culture was centrifuged, and the fermenting capacity tested by using the cells of large volumina, e.g., the cells of 2 liters for the 1- and 2-hour culture. The plate count showed that the cells of

TABLE 5

Effect of the age of cells on the rate of fermentation

AGE OF CULTURE	MILLION CELLS PER CUBIC CENTIMETER		LACTIC ACID PRODUCED	
	Of culture, before centrifugation	Of final cell suspension	In 3 hours	10^{-10} mgm. per cell per hour
hours			*per cent*	
12	379	1,720	0.387	7.5
24	308	1,330	0.279	7.0
24	308	1,220	0.270	7.4
36	142	325	0.108	11.1
48	83	370	0.099	8.9

the culture had not multiplied after 2 hours, and had doubled after 3 hours. There was a definite maximum in the 3-hour culture, but the difference was not nearly as large as that found by the above-cited authors.

Age of cells in hours..........	1	2	3	4	5	6	8	10	36
10^{-10} mgm. per cell per hour...	10.5	16.3	17.7	10.3	9.3	9.2	8.7	6.8	7.4

There seems to be a difference between cells kept young by continuous transfering and those made young by rejuvenation of old cultures.

The values for the cultures older than 12 hours are probably a little too high because the cell numbers begin to decrease, due to aging of the culture. The cells which lose the power to produce colonies may still be able to produce acid (Rahn and Barnes, 1933)

and the computation would ascribe this acid to the viable cells. This is perhaps more plainly visible from table 5 where the number of cells had been determined before and after centrifugation. An estimate of this error does not seem possible.

LONGEVITY

The following experiment was made to test how soon the centrifuged cells lose their vitality. Cells from a 12-hour culture were re-suspended as usual, and the initial plate count of the suspension (3 determinations) was 2500 million per cubic centimeter. After $2\frac{1}{2}$ and $4\frac{1}{2}$ hours at 30°C., the counts showed 1860 and 1890 million per cubic centimeter (6 determinations each).

TABLE 6

Effect of cold storage on viability and fermenting capacity

	MILLION CELLS PER CUBIC CENTIMETER		LACTIC ACID	
	Cells not washed	Cells washed	Cells not washed	Cells washed
			10^{-11} *mgm. per cell per hour*	10^{-11} *mgm. per cell per hour*
Fresh	1,890	1,750	11.6	11.8
1 day at 2°C	1,690	1,720	10.6	11.5
2 days at 2°C	1,650	1,620	8.7	11.4
4 days at 2°C	1,140	1,300	12.6	13.5

The decrease is probably not caused by death, but by partial agglutination of the very slimy culture. One of these cultures was kept at 30°, and the number of viable cells after 24 hours was 1000 million, after 48 hours 760 million per cubic centimeter.

When the original cell concentrate was held at +2°C., and at some later time was warmed to 30° and suspended in the same manner as the above cells, the cells showed very little decrease in viability, and no loss of fermenting capacity for at least 4 days, as may be seen from table 6. The only noticeable effect of cold storage was a delay of 10 to 30 minutes in starting the fermentation. It is therefore feasible to centrifuge the cells on one day and use them for experiments at some later date. All experi-

ments mentioned in this paper have been done with fresh cells, immediately after centrifugation.

EFFECT OF OTHER CARBOHYDRATES

Experiments with carbohydrates other than glucose have shown no acid formation in most cases. The experiments are being continued.

NECESSITY OF PEPTONE

Centrifuged cells show some fermentation without peptone, but when the cells have been thoroughly washed, fermentation in the absence of peptone is very slow or entirely lacking. An investigation on the rôle of peptone has been started.

SUMMARY

The rate of lactic fermentation by streptococci can be measured simply by titration when the cells are obtained by centrifugation and suspended in a buffered glucose solution of pH 7.0. It is important that the cells are young (12 to 24 hours) and more concentrated than normal growth would make them. All cells in these experiments were tested in 2 per cent phosphate buffer + 2 per cent glucose + 0.3 to 0.5 per cent peptone.

The rate of fermentation had its optimal pH at 7.0. Deviation to the alkaline side retarded less than deviation to the acid side. Fermentation took place most rapidly in a buffer containing 4 per cent of phosphates, but the largest amounts of final acidity were produced with 6 per cent.

As long as more than 0.2 per cent of glucose were present, the rate of fermentation was not affected by the sugar concentration.

Sodium lactate in concentration of 1 per cent retarded slightly; 4 per cent reduced the rate to less than half the normal rate.

Continued agitation by a current of air or oxygen retarded fermentation, while nitrogen increased the rate.

Very little difference was found between cells of different age, ranging from 2 hours to 2 days, if the cells were kept young by very frequent transfers. However, if old cells were transferred to a fresh medium, the fermenting capacity was doubled when

the cells came out of lag, and decreased again after the number of cells had doubled.

The centrifuged cells can be stored in a phosphate buffer without glucose at a temperature of about +2°C. for 4 days without loss of fermenting capacity, and with very little loss of viability.

REFERENCES

BAYNE-JONES, S., AND RHEES, H. S. 1929 Bacterial Colorimetry. IV. Relationship of heat production to phases of growth of bacteria. Jour. Bact., 17, 123-140.

RAHN, OTTO, AND BARNES, M. N. 1933 An experimental comparison of different criteria of death in yeast. Jour. Gen. Physiol., 16, 211-229.

SLATOR, A. 1906 The chemical dynamics of alcoholic fermentation by yeast. Jour. Chem. Soc., 89, 128-139.

VIRTANEN, A. I., WICHMANN, E., AND LINDSTRÖM, B. 1927 Ueber die Milchsäuregärung, IV. Z. Physiol. Chemie, 166, 21-38.

WALKER, H. H., WINSLOW, C.-E. A., HUNTINGTON, E., AND MOONEY, M. G. 1931 The physiological youth of a bacterial culture as evidenced by cell metabolism. Jour. Bact., 27, 303-324.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MARYLAND BRANCH

BALTIMORE, DECEMBER 17, 1937

NOTES ON THE HISTORY OF BACTERIOLOGY IN BALTIMORE. *Morris C. Leikind*, Institute of the History of Medicine, Johns Hopkins University.

The beginnings of Bacteriology in Baltimore are practically coincidental with the opening of the Johns Hopkins University in 1876. The biological laboratory headed by Henry Newell Martin fostered investigative work which comprehended biological science in the broadest possible way. So it is not surprising to find Dr. I. E. Atkinson studying the life history of a pathogenic fungus using a pure culture technique in the years 1877-78. Probably the earliest investigator of prominence was George M. Sternberg who early in the 1880's was Fellow by Courtesy in Martin's laboratory. Here, Sternberg completed his researches on disinfectants and his studies on the pneumococcus; and here he trained A. C. Abbott, who later became head of the Hygienic Laboratory at the University of Pennsylvania. In 1885 W. H. Welch came, fresh from the laboratories of Koch and Cohnheim. Bacteriology promptly was given quarters in the laboratory of pathology. Even before the Johns Hopkins Medical School was opened, Welch was conducting classes in the elements of Bacteriology for post graduate medical students. In Welch's laboratory, Mall the anatomist was using bacteria as a tool in anatomic research; Halsted was developing surgical technique on the antiseptic principles laid down by Lister, culminating in the introduction of the rubber gloves.

By 1893 therefore, when the Johns Hopkins Medical School opened its doors, Bacteriology was a flourishing science in Baltimore.

MARYLAND BRANCH

JOHNS HOPKINS CLUB, FEBRUARY 25, 1938

THE BACTERIOLOGY OF CHEESE RIPENING. *L. A. Rogers*, Bureau of Dairy Industry, U. S. Department of Agriculture.

With a few minor exceptions all cheeses owe their characteristic flavor to the action of bacteria or other microorganisms which are naturally present in the milk or are added as pure culture starters. The conditions created by the cheese maker through the control of water and salt content, and the temperatures in the making process and the curing rooms determine the species of bacteria which predominate. In cheese of the Cheddar type *Streptococcus lactis* is encouraged but other varieties have some part in the ripening. In the Swiss cheeses *Streptococcus lactis* is excluded by temperature

conditions and a complicated sequence of bacterial populations occurs in which a delicate balance must be obtained to secure first grade cheese. In Camembert and similar cheeses there is a preliminary lactic fermentation but the characteristic flavor is due to a mold growing on the surface and to enzymatic activity ripening from the surface inwards. Roquefort, Gorgonzola and Stilton, are also mold-ripened with a preliminary lactic fermentation but bacterial action is largely suppressed by a high salt content. By manipulation of the air supply the essential mold is stimulated to develop in the interior of the cheese while other molds are inhibited.

THE VALUE OF THE KLINE FINGER BLOOD TESTS IN THE SERO-DIAGNOSIS OF SYPHILIS. *Charles R. Rein, and Clarice E. Hazay*, 580 Fifth Avenue, New York City.

The Kline flocculation tests for the diagnosis and exclusion of syphilis have been proven to possess ideal sensitivity and specificity. These tests are carried out upon polished surfaces of microscopic slides in chambers of optimal proportions and the results read accurately with ease through the microscope at a magnification of about 120 times. Furthermore the Kline antigen emulsions are satisfactory for use for twenty-four to forty-eight hours. In addition, these tests may be accurately performed in a few minutes with a few drops of blood easily obtainable from a finger puncture or from a bleeding socket following dental extractions. These tests are, therefore, especially adaptable for the serologic detection of syphilis in (1) all donors immediately prior to transfusion, (2) in applicants for insurance, (3) in routine dental practice, (4) in clinic patients, and (5) in children and obese individuals with small difficult veins.

ERRATUM

Vol. 35, No. 5, Page 461

R. H. Broh-Kahn and I. Arthur Mirsky. Studies on anaerobiosis. I. The nature of the inhibition of growth of cyanide-treated *E. coli* by reversible oxidation-reduction systems. Table 2, under the heading "Lactate", anaerobic growth should be – instead of ++++.

BACILLUS PARA-PERTUSSIS: A SPECIES RESEMBLING BOTH BACILLUS PERTUSSIS AND BACILLUS BRONCHISEPTICUS BUT IDENTICAL WITH NEITHER

GRACE ELDERING AND PEARL KENDRICK

Michigan Department of Health, Bureau of Laboratories, Western Michigan Division, Grand Rapids, Michigan[1]

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In connection with a report in 1934 on the results of cough plate examinations for *Bacillus pertussis* (Kendrick and Eldering, 1934a) we noted that all of 136 strains isolated from patients with whooping cough were similar serologically and corresponded with Leslie and Gardner's Phase I (1931). Since then, up to June 1st, 1937, the total number of Phase I cultures has increased to 1498. During that period we have isolated from seven whooping cough patients ten cultures which resemble *B. pertussis* in certain respects but do not conform with all the criteria ascribed to Phase I. Strains similar to these ten were not recovered from over 1500 cough plates from patients with upper respiratory infections other than whooping cough. On the original cough plates from which these organisms were recovered the colonies were typical of *B. pertussis* after 48 hours incubation. Morphology and staining reaction appeared typical of *B. pertussis* and agglutination tests were positive with *B. pertussis* antiserum in low dilution. The plates were reported positive for *B. pertussis* to the attending physicians. However, after longer incubation the colonies on these cough plates were observed to be unusually large and, unlike *B. pertussis*, were found to grow profusely on plain infusion agar and in broth. Recognizing a possible relationship to *Bacillus bronchisepticus*, we tested the cultures

[1] The authors gratefully acknowledge the technical assistance of Gertrude Hodges. For financial assistance, they are indebted to the National Research Council.

against a *B. bronchisepticus* antiserum and found that the organisms were agglutinated to high titer. Unlike *B. bronchisepticus*, however, they were non-motile. Further studies were then carried out, comparing this group of cultures on the one hand with *B. pertussis* and on the other with *B. bronchisepticus*.

SOURCE OF CULTURES

In table 1 are outlined certain details with respect to the source of the cultures.

TABLE 1

Source of cultures

CULTURE NUMBER	PATIENT	AGE	ATTACK DIAGNOSED WHOOPING COUGH		COUGH PLATE POSITIVE FOR "309" GROUP:
			Duration	Severity	
		years	*weeks*		*day of disease*
309	F. B.	12	3	Very light	14th
312	W. R.	3	4	Light	21st
315	J. T.	5	8	Moderate	28th
330					38th
331					45th
344					53rd
595	B. H.	3	4-5	Moderate	8th
609L	B. D.*	7	8	Severe	10th
795	S. S.	4	4	Light	3rd
7010	A. A.	3 months	?	?	13th

* Phase I B. pertussis also recovered.

It is of interest that the first nine cultures from six whooping cough patients were isolated between January 9 and April 19, 1935, a period of a little more than three months, and the tenth culture not until April 14, 1937. Evidence supporting epidemiological relationships is lacking except in one instance. Of the first six patients, F. B. and W. R. lived in the same household. The other four patients lived in scattered sections of the city and had no known contact with each other or with F. B. or W. R. However, there were contacts with other whooping cough patients, from whom we had no cultures for study. It should be noted that from B. D.'s cough plate both Phase I colonies and the

B. pertussis-like colonies were isolated. In the instance of J. T., the *B. pertussis*-like organisms were recovered from four cough plates taken at different stages of the disease.

To indicate relative severity of the attacks we have used the terms severe, moderate, light and very light, as employed in our immunization study and defined in a progress report (1936). According to the criteria used, one of these first six attacks of whooping cough was severe, two were moderate, two were light and one was very light. All but one patient, F. B., whooped, and all were considered to have clinical whooping cough by their attending physicians. The tenth culture was from a plate mailed from outside the city and we received no report on the severity of attack.

STUDY OF CULTURE CHARACTERISTICS

The cultures were studied as to morphology, cultural and biochemical reactions, serological relationships and certain reactions in animals. Since repeated observations indicate that the ten cultures under discussion are of the same group, for convenience, culture 309 is taken as the type of the group and will be referred to by number. The characteristics described for 309 are true for the group, so far as we have been able to determine.

Morphological and growth characteristics. Culture 309 is a small non-motile Gram-negative coccoid bacillus of fairly constant size and shape. In stained preparations it occurs singly or in pairs but seldom in chains. It is aerobic and non-motile. Capsules may be demonstrated by Lawson's method[2]. It grows luxuriantly under aerobic conditions on the usual media with or without the addition of blood. No growth occurs under anaerobic conditions. On Bordet-Gengou medium the colonies are raised, entire, pearly and glistening; this medium is first darkened, but after 24 to 36 hours the darkening is also accompanied by hemolysis. The colonies on plain infusion agar are very similar to those on blood medium and grow rapidly and profusely. After 24 to 48 hours the surrounding medium starts to turn brown, the intensity of the color increasing for four or five days.

[2] G. M. Lawson. Unpublished thesis.

The browning is not affected by light. In beef broth the brown color is also produeed and a viscid sediment occurs, which on agitation rises from the bottom of the tube in a ropy swirl. Litmus milk shows a change toward alkalinity within 48 hours, which increases until the medium is a deep blue after five to seven days.

None of the seven carbohydrates tested (glucose, lactose, sucrose, maltose, mannitol, xylose, and trehalose) is fermented. Indol is not formed. Nitrates are not reduced. Fibrinolytic activity could not be demonstrated. Catalase is produced.

Comparison of culture 309 with B. pertussis and B. bronchisepticus. In stained slide preparations the three cultures appeared so similar that it was desired to eompare their size by actual measurement. This was done by means of photomicrographs

TABLE 2

Comparative lengths of culture 309, B. pertussis and B. bronchisepticus

LENGTH	CULTURE		
	309	*B. pertussis*	*B. bronchisepticus*
microns			
Average	0.52 m.	0.53 m.	0.49 m.
Mean	0.50	0.50	0.50
Range	0.3-0.8	0.3-1.0	0.3-0.8

kindly prepared for us by Dr. H. J. Brown. Stained slides were made from 48-hour cultures on Bordet-Gengou medium, and two fields from each slide photographed, the final magnification representing 1000. For each culture the length of 50 individual bacteria was measured directly on the photograph, and the average and mean obtained as shown in table 2. In view of the difficulties involved in measuring organisms so small, the differences given here are not considered significant. It should be noted that only a few organisms accounted for the extremes of the ranges.

An outline of the morphological and cultural characteristics of culture 309 in comparison with Phase I *B. pertussis* and with *B. bronchisepticus* is given in table 3.

Culture 309 resembles both *B. pertussis* and *B. bronchisepticus*

in morphology, staining reaction, hemolytic zone on Bordet-Gengou medium, catalase production, and in failure to ferment carbohydrates and to produce indol. It is similar to *B. pertussis* but unlike *B. bronchisepticus* in being non-motile and in failing to reduce nitrates. It resembles *B. bronchisepticus* rather than *B. pertussis* in its luxuriant growth on plain infusion agar even when first isolated, rapid production of alkalinity in litmus milk and heavy ropy sediment in broth. It differs from both organisms in the darkening effect on Bordet-Gengou medium and the browning of infusion agar and broth.

TABLE 3

Cultural characteristics: Comparison of No. 309, phase I B. pertussis and B. bronchisepticus

CULTURE	MOTILITY	NITRATE REDUCTION	MEDIUM: Bordet-Gengou: Homolysis	MEDIUM: Bordet-Gengou: Darkening	MEDIUM: Agar	MEDIUM: Broth	MEDIUM: Litmus milk beginning alkalinity
							days
No. 309	−	−	+	+	Heavy growth turning agar brown	Ropy growth with browning	2–4
Phase I *B. pertussis*	−	−	+	−	No growth until adapted	No growth until adapted	12–14
B. bronchisepticus	+	+	+	−	Heavy growth. No pigment	Heavy growth. No pigment	1–2

Agglutination tests. The method of antiserum production and the agglutination technic are the same as given in a report of the Standard Methods Committee on Diagnostic Procedures and Reagents, American Public Health Association (Kendrick, Miller and Lawson, 1935–6). The agglutination titers are expressed in terms equivalent to those employed in the usual 1 cc. test.

In table 4 are given agglutination titers obtained with particular antisera produced against *B. pertussis*, *B. bronchisepticus*, and a culture of the "309" group, respectively.

Titers are given for two different bleedings of each animal to

illustrate the difference in cross agglutination results obtained with different bleedings of the same animal, after more or less intensive immunization. While there is considerable animal variation and a single series of findings is not always reliable, many tests on a rather large number of animals suggest that in general, extended, intensive immunization with one of the three cultures under consideration broadens the serological properties of the serum with respect to the other two and the serum becomes less specific than after a shorter course of immunization. This point may be important in explaining what appear to be dis-

TABLE 4

ANTISERUM		CULTURE USED AS ANTIGEN FOR AGGLUTINATION	AGGLUTINATION TITERS OF BLEEDINGS MADE 5 TO 7 DAYS AFTER LAST INJECTION	
Rabbit number	Homologous culture		3 injections. Total antigen = app. 3 cc.	7 to 10 injections. Total antigen = 12 or more cc.
R193	*B. pertussis* 1445	*B. pertussis*	1:2,500	1:25,000
		"309"	1:100	1:7,500
		B. bronchi.	None	1:5,000
R204	"309" group 315	*B. pertussis*	1:100	1:2,500
		"309"	1:500	1:25,000
		B. bronchi.	1:1,000	1:20,000
R207	*B. bronchiseptica* 214	*B. pertussis*	1:100	1:15,000
		"309"	None	1:2,500
		B. bronchi.	1:2,500	1:25,000

Note: Homologous titers are underlined.

crepancies in the findings of different workers, in addition to the possibility of somewhat different strains having been chosen by them for study. For example, when Ferry and Noble (1918) and Ferry and Klix (1918) reported on the close relationship between *B. pertussis* and *B. bronchisepticus*, they observed that *B. pertussis* antiserum did not agglutinate *B. bronchisepticus*. We have found this true with certain bleedings of our animals, as in R-193 (table 4) after three injections. In other bleedings, as R-193 after 10 injections, the serum gave a positive agglutination with *B. bronchisepticus*.

From the results with a series of rabbits for each antiserum, the average cross agglutination results with bleedings after 9 to 11 injections were as follows. *B. pertussis* antiserum gave titers with "309" and *B. bronchisepticus* which were about 20 per cent of the average homologous titer 1:25,000. The "309" antiserum agglutinated *B. pertussis* to 5 per cent of the average homologous titer 1:25,000, and *B. bronchisepticus* to 40 per cent of the titer. *B. bronchisepticus* antiserum agglutinated *B. pertussis* to 20 per cent and "309" to 65 per cent of the homologous titer of 1:13,750.

In general, the findings suggest a common antigenic component for *B. pertussis*, "309" group and *B. bronchisepticus*.

Agglutinin-absorption tests. The antigenic relationships between the three organisms were studied further by agglutinin absorption. Briefly, the serum for absorption was diluted 1:10 and mixed with a previously determined absorbing dose of washed organisms, usually 0.1 to 0.2 cc. of packed bacteria. The mixture was incubated two hours in a water bath at 37°C., and placed in the icebox overnight. The serum was then clarified by centrifugation, and agglutination tests were made, using the rapid method.

The findings in several series of agglutinin-absorption tests in general confirm those of the one series tabulated in table 5 and expressed graphically in table 6.

The results indicate that although the three organisms are related as shown also by the cross agglutination reactions, they are not identical. Each of the three cultures completely removes agglutinins for itself from each of the three antisera. Each culture removes the agglutinins for each of the other two cultures also from the antiserum produced against itself; it only partially absorbs these heterologous agglutinins from the sera produced against the other two cultures, respectively. In those antisera which gave relatively high titers after absorption, an attempt was made to complete the removal of agglutinins by repeated absorptions. Only slight or no further reductions in titer were produced by this method.

Reactions in animals. Certain reactions produced in animals by living cultures of *B. pertussis* were considered in a previous

report (1934b). Mice inoculated intraperitoneally with a five billion dose of *B. pertussis* usually die within two to four days and at autopsy show extensive hyperemia of the peritoneal wall, infiltration of the lymph glands, and the presence of an extremely mucoid exudate in the peritoneal cavity. The organisms can be recovered from this exudate and, with less regularity, from the heart's blood. Similar results in mice were obtained with culture 309 and it may be mentioned that after serial passage through

TABLE 5

Agglutinin-absorption results in one series of tests

ANTIGEN USED FOR ABSORPTION OF SERUM	ANTISERA USED IN AGGLUTINATION TESTS								
	Rabbit 204			Rabbit 193			Rabbit 223		
	Homologous culture								
	"309" group—315			*B. pertussis*—1445			*B. bronchisepticus*—L279		
	Agglutination titers with different culture suspensions as antigen—Agglutination antigens								
	315	1445	L279	315	1445	L279	315	1445	L279
None (not absorbed)....	1:40T	1:5T	1:1T	1:25T	1:35T	1:2.5T	1:7.5T	1:500	1:25T
"309" group—315.	Neg.	1:100	Neg.	1:100	1:20T	Neg.	Neg.	1:100	1:20T
B. pertussis—1445..... ..	1:25T	Neg.	Neg.	1:1T	Neg.	Neg.	1:7.5T	Neg.	1:25T
B. bronchisepticus—L279.......	1:25T	1:1T	Neg.	1:7.5T	1:15T	Neg.	1:1T	Neg.	Neg.

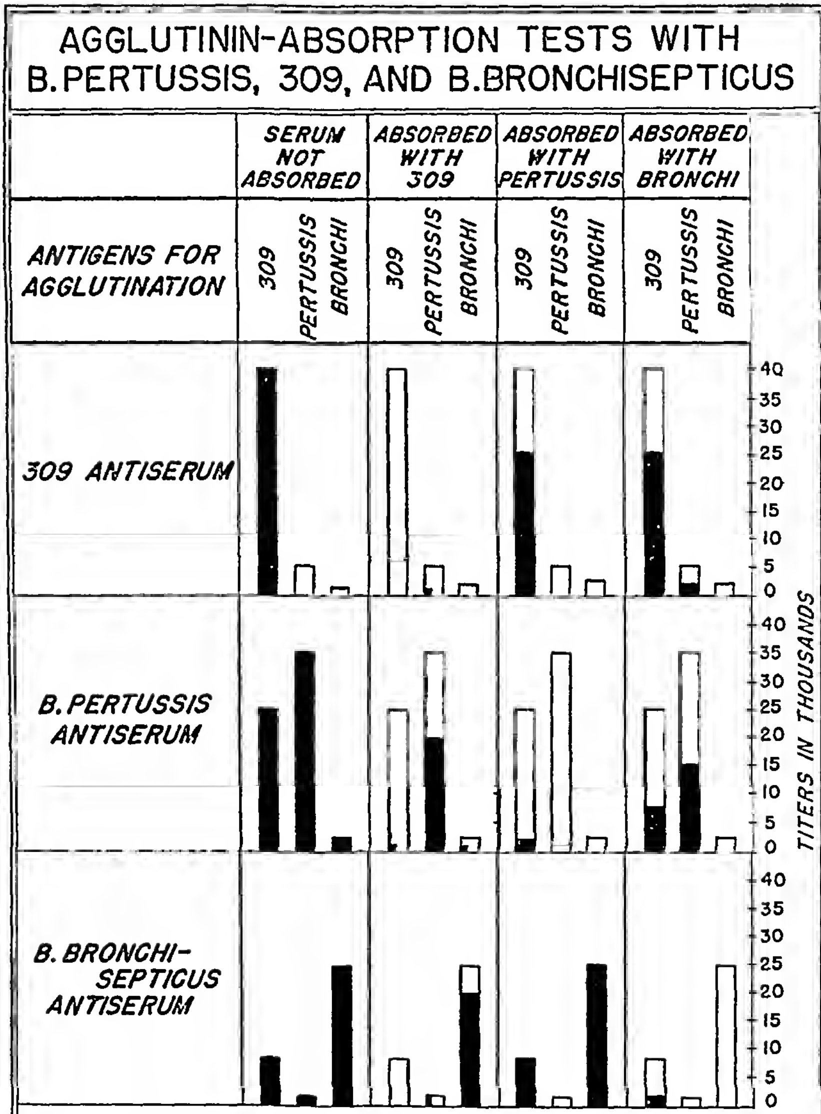
Note: Neg. = <1:100; T = 1,000 i.e. 1:25T = 1:25,000; homologous titers are underlined.

three mice, the culture retained all its original characteristics. *B. bronchisepticus*, injected into mice produced results comparable with those described for *B. pertussis* and culture 309.

Intradermal inoculations in rabbits of 0.1 cc. of a 1 billion suspension of *B. pertussis*, culture 309 and *B. bronchisepticus* respectively, all produced the reaction of hemorrhagic necrosis characteristic of *B. pertussis*.

In their study of the opsonocytophagic reaction in pertussis,

TABLE 6



Kendrick, Gibbs and Sprick (1937) found that in general all three cultures gave strong reactions with the blood of an individual immunized against *B. pertussis*.

DISCUSSION

Significance of the "309" cultures. In considering the significance of this group of organisms, typified by culture 309, the question arises as to the frequency with which they occur and their distribution. In our own experience they are relatively infrequent; only ten such cultures were isolated over a period of five years during which time 1498 cultures of *B. pertussis* were obtained. Miller[3] working with Madsen at the State Serum Institute in Copenhagen isolated two cultures which he describes as similar to our 309 group. These two cultures are referred to as "green strains" by Krarup (1933). Miller isolated two additional "green strains" in California and we are indebted to him for sending us one of them for study. It has all the characteristics of our 309 group. One of the California cultures was isolated from a child who coughed for three weeks but did not whoop, although her cough was described as paroxysmal. The other was recovered from a severe case of whooping cough from which Phase I *B. pertussis* was also obtained. Lawson,[4] who has kindly examined culture 309, states that he has isolated a culture having the same characteristics from a patient in Louisville, Kentucky. It would be of interest to know if other workers have isolated similar cultures.

The finding of such a homogeneous group of cultures, though they are relatively few in number, has both theoretical and practical interest. These strains might be looked upon as a result of the dissociative process in *B. pertussis*. This is suggested by the occasional finding of both *B. pertussis* and a culture of the 309 group in the same patient. The cultures of this group however are not the same as any of the old laboratory strains which we have studied. In our experience, old strains of *B. pertussis* which have lost their Phase I characteristics have a morphology quite different from any of the three cultures under discussion—the organisms are longer and there is a tendency to thread formation. Also, they do not show browning of the medium, do not

[3] J. J. Miller. Personal communication.
[4] G. M. Lawson. Personal communication.

grow as profusely or easily as do the 309 group and are relatively inactive in animals. Perhaps the cultures of the 309 group bear a relationship to Phase I. *B. pertussis* more like that of the *Salmonella* group to *Eberthella typhi.* Whatever the status of the group, the findings with respect to it strengthen the accumulating evidence of the close relationship between *B. pertussis* and *B. bronchisepticus.*

A question naturally arises whether these organisms of the 309 group bear a similar relation to the disease whooping cough to that of *B. pertussis;* and if so, whether any protective properties possessed by a vaccine prepared from either *B. pertussis* or the 309 group would be effective against infection by the other organism. The data at hand are insufficient to answer these questions.

Note on classification. In our various references to pertussis bacilli we have avoided the use of the generic term *Hemophilus* so widely used at present, suggesting a close relationship to *H. influenzae.* The cultural characteristics of pertussis bacilli give fully as good, if not better, reasons for considering them cogeneric with *Bacillus bronchisepticus*—an organism placed by some in the genus *Alcaligenes* and by others in *Brucella.* Believing that the systematic position of the pertussis organism requires further elucidation, we have used the designation *Bacillus pertussis* without prejudice to any other generic terminology which may later seem desirable.

The "309" cultures described in this report definitely cannot be placed in the genus *Hemophilus* since they grow readily on ordinary blood-free media. For convenience of reference we suggest the name *Bacillus para-pertussis,* until we are able to recognize a clear basis for an appropriate generic designation of the *pertussis-bronchisepticus* group of organisms.

SUMMARY

Ten cultures forming a homogeneous group were isolated from seven whooping cough patients by the cough plate method. They have been studied according to morphology, cultural and serological characteristics, and certain reactions in animals.

While closely related antigenically to both *Bacillus pertussis* and *Bacillus bronchisepticus*, these cultures are identical with neither. Their significance in connection with the disease whooping cough is not yet clear. For convenience of reference, the name *Bacillus para-pertussis* is used for the organisms described.

REFERENCES

FERRY, N. S., AND KLIX, H. C. 1918 Studies relative to the apparent close relationship between *Bact. pertussis* and *B. bronchisepticus*, II. J. Bact., 3, 197–199.

FERRY, N. S., AND NOBLE, ARLYLE 1918 Studies relative to the apparent close relationship between *Bact. pertussis* and *B. bronchisepticus*, I. J. Bact., 3, 157–171.

KENDRICK, P., AND ELDERING, G. 1934a Cough plate examinations for *B. pertussis*. A. J. P. H., 24, 309–318.

KENDRICK, P., AND ELDERING, G. 1934b A study of *B. pertussis* cultures by means of animal inoculation. J. Bact., 27, 97 (Abstract).

KENDRICK, P., AND ELDERING, G. 1936 Progress report on pertussis immunization. A. J. P. H., 26, 8–12.

KENDRICK, P., GIBBS, J., AND SPRICK, M. 1937 The opsonocytophagic test in the study of pertussis. Jour. Inf. Dis., 60, 302–311.

KENDRICK, P., MILLER, J. J., AND LAWSON, G. M. 1935–1936 Tentative methods for the bacteriological diagnosis and control of whooping cough. Report of Referee. A. P. H. A. Year Book, pp. 200–206.

KRARUP, N. B. 1933 Réactions intracutanées dans la coqueluche. Comp. Rend. Soc. de Biol., 115, 85–88.

LESLIE, P. H., AND GARDNER, A. D. 1931 The phases of *Haemophilus pertussis*. J. Hyg., 31, 423–434.

CELL INCLUSIONS AND THE LIFE CYCLE OF RHIZOBIA

I. M. LEWIS

Department of Botany and Bacteriology, the University of Texas

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Few groups of non-pathogenic bacteria have held such great interest or have been so thoroughly studied as the rhizobia. The recent monograph by Fred, Baldwin, and McCoy (1932) affords a full review of the voluminous literature. Excellent reviews of the early period of study were given by Atkinson (1892) and by Erwin F. Smith (1911). No very critical review has appeared on the life cycle.

Prior to isolation and cultivation of pure cultures, belief in a more or less complex life history prevailed. The earliest workers who studied root nodules were confused by the presence of hypha-like infection threads, and bacteroids within the root tissue. They generally regarded the bacteroids as spores produced by a budding mycelium. Accordingly the organism was regarded as a fungus and a complex life history was assumed.

With the isolation and study of pure cultures by Beijerinck (1888) many of the previous notions were abandoned but new discoveries seemed to support the belief in a life history different from that which was accepted for other species of bacteria. Beijerinck noted that pure cultures consisted of rod-like cells 1 by 4μ, coccoid cells 0.18 by 0.4μ, and large vacuolated bacteroids. He introduced the term, swarmers, for the smaller cells. This term has since come to occupy a prominent place in discussions of the life cycle.

The belief that rhizobia reproduce by small bodies borne within large vacuolated cells has been advocated by many workers. This idea seems to have originated in the early work of Morck (1891) who studied the bacteroids from root nodules of many legumes. Hiltner (1898) was probably the first to speak

of bacteroids as sporangia. Hartleb (1900) stated that bacteroids liberate spore-like bodies which develop into swarm cells. Schneider (1894) thought that bacteroids produce endospores. He abandoned this view (1902) but maintained that spore-like bodies, sporoids, are produced within the bacteroids. De Rossi (1907) and Greig-Smith (1899) believed that several cells arise from each bacteroid.

The life history of rhizobia has been studied more recently by Löhnis and Smith (1916), Löhnis and Hansen (1921), Bewley and Hutchinson (1920), Thornton and Gangulee (1926), Gangulee (1926), Káš (1927), Gibson (1928), and Schönberg (1929). Reproduction by gonidia has been accepted by all these writers and in some cases symplasm and conjunction have been described.

According to Bewley and Hutchinson (1920) rhizobia reproduce by formation of cocci within the rod-like cells. They describe five types of cells and the sequence of occurrence in the life cycle. *Banded rods* liberate *small non-motile cocci* which increase in size to form *larger non-motile cocci*. These elongate to form *small ovoid motile cells* and by further development change to *elongated, unbanded, motile rods*. The stainable substance rounds off to form *small cocci* thus completing the cycle. External conditions were found to influence the formation of cocci within the rods and change to the motile condition but the course of development was regarded as inherent and normal.

Thornton and Gangulee (1926) and Gangulee (1926) confirmed the occurrence of these five cell types. However, according to their observation cocci may become flagellated before release from the mother cell. The life cycle described by Bewley and Hutchinson has been accepted by various writers of recent books such as Topley and Wilson (1936), Frobisher (1937), and Russell (1937).

CELL INCLUSIONS

Although a belief that rhizobia reproduce by gonidia has generally prevailed, there are certain recorded observations concerning the cell structure and viability of bacteroids which indicate a possibility that this belief is not well founded. It is a

matter of common observation that bacteroids from nodules or cells from old cultures stain poorly with anilin dyes, and when stained show a structure which has been variously described as banded, barred, vacuolated, granulated or alveolar. This is very good presumptive evidence for the presence of non-stainable, lifeless cell inclusions within the cytoplasm and suggests the probability that the belief in reproduction by gonidia is based on an erroneous interpretation of the cell structure.

Various writers have referred to the fact that bacteroids contain highly refractive granules which are not stained by anilin dyes. Atkinson (1892) noted such granular bodies and identified them tentatively as condensed masses of protein. He wrote as follows:

> On staining bacteroids from tubercles of *Medicago denticulata* with fuchsin they present an interrupted stain, simulating in this respect the rods of *Bacillus tuberculosis.* It is quite likely that the difficulty experienced in staining these objects in the tubercles has led some to describe the stained portions as spores.

Lundstrom (1888) observed that bacteroids from nodules which had been in water for some time were filled with refractive granules which he regarded as inclusions consisting of condensed protein.

More definite studies on these granular bodies have been recorded. Prazmowski (1890) studied bacteroids from various species. He observed refractive granules and attempted to determine their nature by various microchemical tests. He found that the granules resisted the action of alkali, were stained brown with iodine, red with concentrated sulphuric acid, and failed to stain with anilin dyes. For these reasons he regarded the bodies as protein. This view was criticized by Frank (1892) who maintained that the identification by Prazmowski could not be valid since the bodies should stain with anilin dyes if they consist of protein. He regarded the bodies as amylodextrin.

The most complete study of the chemical nature of bacteroidal granules is that of Möller (1892). He objected to the conclusions of both Frank and Prazmowski concerning the nature of the

substance. His conclusions were based on solubility tests, and staining reactions. He showed that the granules were insoluble in cold dilute potassium hydroxide, in cold or boiling concentrated ammonia, in hot ethyl alcohol and amyl alcohol, in ether, benzene or carbon bisulphide. No vaporization or other changes occurred on careful heating over a flame. The granules were found to be soluble in chloroform, acetone, glacial acetic acid, clove oil and less easily in benzol. He interpreted these reactions as indicative of a fatty or waxy substance corresponding most nearly to cholesterin but not identical. In a later publication Frank (1892) abandoned the idea of carbohydrate composition and supported Möller's conclusion.

Heinze (1905) noted granules in bacteroids from nodules of pea, vetch, broad bean, garden bean, clover, and alfalfa. The bodies stained red-brown with iodine and were identified as glycogen. Marie P. Löhnis (1930) observed granular bodies in bacteroids of pea plants grown on a sterilized medium but did not determine their chemical nature. Müller and Stapp (1926) showed by chemical analyses that bacteroids contain fatty acids or esters of fatty acids and glycerol or a mixture of these, and a wax-like substance probably cholesterin. Glycogen was not found.

Since these early reports the presence of cell inclusions in rhizobial cells appears to have been generally disregarded. Most writers have designated unstained portions of the cell as vacuoles and stainable portions as gonidia or spores. Gibson (1928) called attention to the nature of non-stainable bodies in banded cells. He concluded that these are not vacuoles but non-stainable gonidia containing a fat-like substance.

CYTOLOGICAL OBSERVATIONS

The observations on cell structure and reproduction to be recorded here are based on a study of several species of *Rhizobium* cultivated in a variety of culture media and examined by various methods during the growth cycle. Pure cultures of *R. meliloti*, *R. lupini*, *R. trifolii*, *R. leguminosarum* and *R. japonicum* were supplied by Dr. E. B. Fred from the extensive Wisconsin collec-

tion. The yeast-water mannitol medium described by Waksman and Fred (1928) was employed as a culture medium for most purposes. This medium was varied in some cases by substituting sucrose or glucose for mannitol. Soil extract, soil-extract agar, bouillon, beef-extract peptone agar, and each of these containing mannitol, glucose or sucrose were also employed as culture media.

Microscopic examinations were made of cells from young and old cultures. The preparations included nigrosin and congo-red films, heat-fixed films stained with various anilin dyes, hanging drop and flat wet mounts with special staining methods for fat inclusions.

Since most writers have attached great importance to the banded or vacuolated cells as the bearers of gonidia, the structure and the conditions which favor formation of this cell type in cultures received special attention. Cells from old cultures on yeast-water mannitol agar contain highly refractive spherical granules which are not stained by aqueous solutions of anilin dyes. They are stained intensely by the naphthol-blue method of Dietrich and Liebermeister (1902), by the several methods described by Eisenberg (1909), and by Sudan III. Accordingly, they are regarded as cell inclusions of a fat-like substance. This identification is supported by the early work of Möller (1892) and by the recent study of Hopkins and Peterson (1930), who studied ether and chloroform extracts by precise macrochemical methods. Their results show that about 20 per cent of the dry weight is soluble in chloroform.

Fat bodies are present in practically all cells of the various species studied after the cultures reach an age of three or four days and may be observed in many cells in 24-hour cultures. The number of bodies varies with size of the cells. Long rods may contain four to six while short rods contain one or two. Large inflated bacteroids contain many fat bodies, the cytoplasmic content is reduced to a mere reticulum, and the structure appears alveolar. When stained with aqueous solution of anilin dyes, the fat-containing cells appear to be vacuolated and banded since unstained fat bodies, which may equal the diameter of the cell, alternate with stained masses of cytoplasm (figs. 1 and 3).

The principal factor which favors formation of fat bodies is the composition of the culture medium. They were more abundant in media containing mannitol, sucrose or glucose. It is impossible to agree with Bewley and Hutchinson (1920) that formation of banded cells depends on the absence of carbohydrates. According to their conclusions such cells are more abundant in soil extract without carbohydrate and do not occur in carbohydrate media until depletion of the carbon compound has occurred.

The occurrence of banded cells has been offered as conclusive proof of reproduction by gonidia. According to this idea the cell content becomes broken up into two or more stainable bodies which escape from the mother cell as very minute non-motile cocci or pre-swarmers analogous to asexual spores of the algae and fungi.

The nature of the process which causes the banded condition in cells of rhizobia is not such as we would expect to occur in a true method of reproduction. The formation of reproductive cells, zoospores and gametes, within the body of a mother cell is a common method of reproduction in many of the algae and fungi and has been advocated for many species of bacteria. But such cells originate by cleavage of the protoplast and are, therefore, true cells. The stainable bands in cells of rhizobia are not formed in this manner as has been shown above and the protoplast is not divided as the term is properly used. There is a single protoplast containing fat bodies. This gives a superficial resemblance to division of the cell contents to form small coccus-like reproductive bodies. This condition occurs in other fat depositing species of bacteria such as *Bacillus mycoides* and *Bacillus anthracis*. Comparing the photographs of *Rhizobium meliloti* (fig. 1) and *B. mycoides* (fig. 2) it is seen that the structure is identical. It may be noted that the stained bands are generally convex while the clear fat bodies are spherical or oval. In very old cells in which autolysis has set in, the cytoplasmic bodies round off and bear a superficial resemblance to cocci. In advanced stages of cell disintegration the wall ruptures and the contents are set free. The protoplast in disintegration is broken

up into separate particles which now stain poorly, and have the appearance of degeneration products.

Additional evidence that banded cells produce gonidia has been offered by Gangulee (1926) who reported the development of flagella on the bodies while still enclosed in the mother cell. The diagramatic drawings presented by Thornton and Gangulee (1926) to illustrate such flagellation are not convincing. The drawings do not show internal flagellated bodies but banded rods with flagella borne externally as in the case of unbanded rods. Examination of rhizobial cells containing fat bodies reveals the fact that such cells are frequently motile. The same is true of other fat-depositing motile species such as *Bacillus cereus*. The banded flagellated rods are doubtless cells which have deposited fat bodies while still retaining their original flagella.

Ledingham (1931) suggested that the banded rods might be due to some form of multiple fission. A somewhat similar view was held by Schneider (1902). Thornton (1931) replied to Ledingham that "owing to the small size of the organism it is difficult to be sure whether transverse walls are formed between the coccoid bodies before their release. But it is probable that the bodies are released from inside, since the empty and disintegrating mother cells can often be seen in preparations made when the cocci are appearing." It appears to the writer that both of these conceptions are based on erroneous interpretations of the cell structure.

The banded condition appears to bear no relation to a method of reproduction when the real cause of banding is understood. There might be some who would maintain that the fat bodies are themselves non-stainable gonidia impregnated with fat-like material but containing also living substance. This matter has been discussed in my previous paper on the cell inclusions of *Azotobacter* (1937). Similarly it could be maintained that the stainable masses although conditioned by the presence of fat bodies are segments of the protoplasm and should be capable of growth when set free from the cell. This contention must not be passed lightly for it would apply equally as well to other fat-depositing species including many members of the genus *Bacillus* and probably the mycobacteria as well.

Such an extreme view is not compatible with previous knowledge of the process of reproduction and is not supported by any known facts. Many workers have attempted to determine whether or not the bacteroids are viable either by liberation of living internal units or by buds broken directly off the parent cell. This subject has been fully reviewed by Almon (1933) who made a careful study by means of single-cell isolation. Her results show that of 411 bacteroids isolated from nodules only one gave evidence of growth in various media favorable for multiplication. This one case was discounted on the grounds of experimental error. A total of ten vacuolated cells were isolated from old cultures and planted in yeast-water mannitol solution. Growth occurred in one case only. Again the result was discounted since some difficulty was encountered in recognizing bacteroids in the unstained condition. Müller and Stapp (1925) observed individual bacteroids microscopically during an incubation period of 72 hours but saw no changes although the medium was favorable for reproduction. Bazarewski (1927) believed that very young bacteroids might transform themselves into simple rods such as those from which they arose but did not reproduce otherwise. It appears, therefore, that the evidence, both cytological and cultural, is directly opposed to the theory of viable units borne within the so-called vacuolated cells.

Old cultures of rhizobia are characterized by extreme pleomorphism. A most thorough study of this subject was made by Buchanan (1909). His camera-lucida drawings show that old cultures consist of a mixture of cell types which are extremely variable as to size and form. He concluded, however, that this does not signify a life cycle through which the organism must pass but is due to external conditions.

The occurrence of pleomorphic cell types has been reported in various bacillary species by many workers including Henrici (1928), Stapp and Zycha (1931), Lewis (1932), who have discussed the possible relation of such pleomorphic cells to a definite life cycle.

It is not necessary to assume that the coccus-like cells in old cultures of rhizobia are gonidia or that large granulated cells are

gonidiangia. In the light of our knowledge of the cell structure, such an assumption is not tenable. It is well known that the form and size of cells is a function of the rate of growth and rate of division. As the growth rate slows down, cell division may continue at an unaltered rate and some of the cells become smaller and smaller. This results in the formation of short ovoid cells and pseudococci. In some cases the cells appear to lose the capacity for cell division while retaining capacity for growth and deposition of fat bodies and become transformed into elongated banded rods and swollen bacteroids. It may also be observed that many of the small ovoid cells and cocci are non-motile, contain tiny fat bodies, and must be regarded, therefore, as old mature vegetative cells. The process may be reversed by planting cells from old cultures in a fresh culture medium.

A study of the growth phases of rhizobia reveals nothing unusual. When cells from old cultures are transplanted to fresh media and examined during the early and late phases of growth an orderly sequence in development occurs. Cells from young cultures are characterized by absence of fat bodies and presence of flagella. The cytoplasm stains deeply and uniformly with aqueous solutions of anilin dyes. Some fat bodies appear within 24 hours in culture media containing glucose, sucrose or mannitol. As the culture develops there is a tendency toward uniformity in shape and size of the cells which is most strongly marked in cultures after 18 to 24 hours on mannitol yeast-water agar. Cells from 48 hour cultures contain fat bodies but in many cases have retained motility and are capable of cell division. With further age motility is lost and the pleomorphic cell types appear. Cultures after seven days consist of a mixture of long and short rods, pseudococci, small ovoid cells, branched rods and swollen granulated bacteroids. In still older cultures many of the cells have become autolysed and broken down with liberation of fat bodies and partially disintegrated masses of cytoplasm.

The sequence of events in cultures of rhizobia is similar to that already known for such species as *Bacillus mycoides*, *Bacillus megatherium* and others. The evidence does not appear sufficient to warrant the conclusion that the observed phenomena are due

to a cyclogenic life history. The term, growth cycle, is preferable since it does not imply a cyclogenic life history based on specialized reproductive cells but indicates a repetitive sequence in the growth phases of cultures when transplanted at appropriate intervals. The transition from motile to non-motile condition and back to motile, is not unusual since it occurs in other motile species. There is, however, no reason to doubt that these transitions may be influenced by the culture medium and other conditions of cultivation and correlation with phases of growth is to be expected. The formation of pseudococci and ovoid cells in old cultures and the transition back to rods when placed in fresh media is due to response to changing conditions for growth and not to an inherent method of reproduction other than cell division. The capacity to deposit fat bodies is inherent but it is influenced by the culture medium and correlates with the age of the culture.

The view set forth above is not different from that of previous writers so far as the cell types and their sequence in the "upgrowth and downgrowth" in cultures is concerned but is directly opposed to some previous interpretations of cell structure and reproduction by gonidia and spores.

SUMMARY

Various species of *Rhizobium* have been studied by cytological and cultural methods. The cells deposit fat bodies which are not stained by anilin dyes. The banded or barred condition of cells stained with anilin dyes is due to stained areas of cytoplasm which alternate with unstained fat bodies. The fat bodies are regarded as lifeless cell inclusions. There seems no reason to consider the stainable cytoplasmic areas as gonidia or viable units which perform a reproductive function.

The morphological and cytological changes which occur when cells from old cultures are transplanted to fresh media correlate with the phases of growth. Such changes are cyclic when transplants are made at suitable time intervals and the term growth cycle is, therefore, appropriate to designate the series of repetitive

changes. The life history is not cyclogenic in the sense that special reproductive cells, gonidia or spores, are formed in the process of reproduction.

REFERENCES

Almon, Lois 1933 Concerning the reproduction of bacteroids. Centr. f. Bakt., II Abt., **87**, 289–297.

Atkinson, G. F. 1893 Contribution to the biology of the organism causing leguminous tubercles. Bot. Gaz., **18**, 157–166, 226–237 and 257–266.

Bazarewski, S. 1927 Badania nad bakteroidami. Roczniki Nauk Rolniczych i Lesnych., **17**, 1–34.

Beijerinck, M. W. 1888 Die Bacterien der Papilonacean knöllchen. Bot. Ztg., **46**, 726–735, 741–750, 757–771.

Bewley, W. F., and Hutchinson, H. B. 1920 On the changes through which the nodule organism (*Ps. radicicola*) passes under cultural conditions. Jour. Agr. Sci., **10**, 144–162.

Buchanan, R. E. 1909 The bacteroids of *Bacillus radicicola*. Centrabl. f. Bakt. etc. II Abt., **23**, 51–91.

Dietrich, A., and Liebermeister, G. 1902 Sauerstoffubertragende Körnchen in Mitzbrandbacillen. Centrblt. f. Bakt., I Abt., Orig., **32**, 858–865.

Eisenberg, P. 1909 Ueber Fetteinschlusse bei Bakterien. Centralblt. f. Bakt., I Abt., Orig., **48**, 257–274.

Frank, B. 1892 Ueber den Dimorphismus der Wurzelknöllchen der Erbse. Ber. Deut. Bot. Gesell., **10**, 170–178.

Frank, B. 1892 Ueber Moller's Bemerckungen bezüglich der dimorphen Wurzelknöllchen der Erbse. Ber. Deut. Bot. Gesell., **10**, 390–395.

Fred, E. B., Baldwin, I. L., and McCoy, E. 1932 Root nodule bacteria and leguminous plants. University of Wisconsin Studies in Science, Number 5, 1–343.

Frobisher, M. 1937 Fundamentals of Bacteriology. W. B. Saunders Co., Philadelphia and London.

Gangulee, N. 1926 Studies on the lucerne nodule organism (*B. radicicola*) under laboratory conditions. Ann. Appl. Biol., **13**, 360–373.

Gibson, T. 1928 Observations on *B. radicicola*, Beijk. Jour. Agr. Sci., **18**, 76–89.

Greig-Smith, R. 1899 The nodule organism of the Leguminosae. Proc. Linn. Soc. N. S. Wales, **26**, 653–673.

Hartleb, R. 1900 Die Morphologie und systematische Stellung der sogenannten Knöllchenbakterien. Chem. Ztg., **24**, 887–888.

Heinze, B. 1905 Einige Berichtigungen und weitere Mitteilungen zu der Abhandlung: "Ueber die Bildung und Wiederverarbeitung von Glykogen durch niedere pflanzliche Organismen." Centralbl. f. Bakt. II Abt., **14**, 75–87.

Henrici, A. T. 1928 Morphologic Variation and Rate of Growth. Springfield and Baltimore, Charles C. Thomas.

HILTNER, L. 1898 Ueber Enstehung und physiologische Bedeutung der Wurzelknöllchen. Forstl. Naturw. Ztschr., 7, 415–423.

HOPKINS, E. W., AND PETERSON, W. H. 1930 The nature of the fat-like constituents of the alfalfa root nodule bacteria. Jour. Bact., 19, 9–10.

KAS, V. 1927 Ueber die Entwicklungszyklen der Knöllchenbakterien. Vestnik. Ceskoslov. Akad. Zemedetcke, 3, 584–588.

LEDINGHAM, J. C. G. 1931 Discussion on the life-cycles of bacteria. Fifth Internatl. Bot. Congress, Report of Proc., page 42. University Press, Cambridge.

LEWIS, I. M. 1932 Dissociation and life cycle of *Bacillus mycoides*. Jour. Bact., 24, 381–414.

LÖHNIS, F., AND SMITH, N. R. 1916 Life cycles of bacteria. Jour. Agr. Res., 23, 401–432.

LÖHNIS, F., AND HANSEN, R. 1921 Nodule bacteria of leguminous plants. Jour. Agr. Res., 20, 543–556.

LÖHNIS, MARIE P. 1930 Investigations upon the ineffectiveness of root-nodules on Leguminosae. Centrablt. f. Bakt., etc., II Abt., 80, 342–368.

LUNDSTROM, A. N. 1888 Ueber Mykodomatien in den Wurzeln de Papilionaccan. Bot. Centrablt., 33, 185–188.

MÖLLER, H. 1892 Bemerkungen zu Frank's Mitthellung ueber den Dimorphismus der Wurzelknollchen der Erbse. Ber. Deut. Bot. Desell., 10, 242–249.

MORCK, D. 1891 Ueber die Formen der Bakteroiden bei den Einzelnen Spezies der Leguminosen. Inaug. Diss., Leipzig, 44 pp.

MÜLLER, A., AND STAPP, C. 1925 Beiträge zur Biologie der Leguminosenknollchen bakterien mit besonderer Beruecksichtigung ihrer artverschiedenheit. Arb. Biol. Reichsanst. Land. u. Forst., 14, 455–554.

PRAZMOWSKI, A. 1890 Die Wurzelknöllchen der Erbse. Landw. Vers. Sta., 37, 161–238.

ROSSI, G. DE 1907 Ueber de Mikroorganismen welche die Wurselknöllchen der Leguminosen erzeugen. Centrbl. Bakt., etc., II Abt., 18, 289–314 and 481–488.

RUSSELL, E. J. 1937 Soil Conditions and Plant Growth. 7th Ed. Longmans, Green and Co., London.

SCHNEIDER, A. 1894 Beitrag zur Kentniss der Rhizobien. Ber. Deut. Bot. Gesell., 12, 11–17.

SCHNEIDER, A. 1902 Contribution to the biology of rhizobia. Bot. Gaz., 34, 109–113.

SCHÖNBERG, L. 1929 Untersuchungen ueber des Verhalten von Bact. radicicola Beij. gegenüber Verschiedenen Kohlehydraten und in Milch. Centralbl. f. Bakt., II Abt., 79, 206–221.

STAPP, C., AND ZYCHA, H. 1931 Morphologische Untersuchungen an *Bacillus mycoides* in Beitrag zur Frage des Pleomorphismus. Archiv. f. Microbiol., 2, 493–536.

TOPLEY, W. W. C., AND WILSON, G. S. 1936 Principles of Bacteriology and Immunity. Second edition, 373–376. Wm. Wood and Co., Baltimore.

THORNTON, H. G. 1930 A System of Bacteriology in Relation to Medicine, I. His Majesty's Stationery Office, London.

THORNTON, H. G., AND GANGULEE, N. 1926 The life-cycle of the nodule organism, *Bacillus radicicola* (Beij) in soil and its relation to the infection of the host plant. Proc. Roy. Soc. Ser. B., 99, 427–451.

THORNTON, H. G. 1931 Discussion on the life-cycles of bacteria. Fifth Internatl. Bot. Congress, Rept. of Proc., page 42. University Press, Cambridge.

WAKSMAN, S. A., AND FRED, E. B. 1928 Laboratory Manual of General Microbiology. McGraw-Hill Book Company, New York.

PLATE 1

Fig. 1. Stained cells of *Rhizobium meliloti* from root nodule of alfalfa showing clear fat bodies and stained bands of cytoplasm. Photographed by E. B. Fred.

Fig. 2. Cells from a culture of *B. mycoides* grown on glucose agar and stained by Gram's method. The clear unstained fat bodies alternate with stained cytoplasm.

Fig. 3. Diagramatic drawings illustrating the appearance of unstained cell of *Rhizobium trifolii* and the same with different staining methods. (*a*) Unstained showing refractive granules. (*b*) Fat bodies stained by the naphthol blue method. The cytoplasm is colorless. (*c*) Stained with methylene blue. The unstained fat bodies alternate with stained bands of cytoplasm. (*d*) Flagellated banded cell according to Thornton and Gangulee. See text.

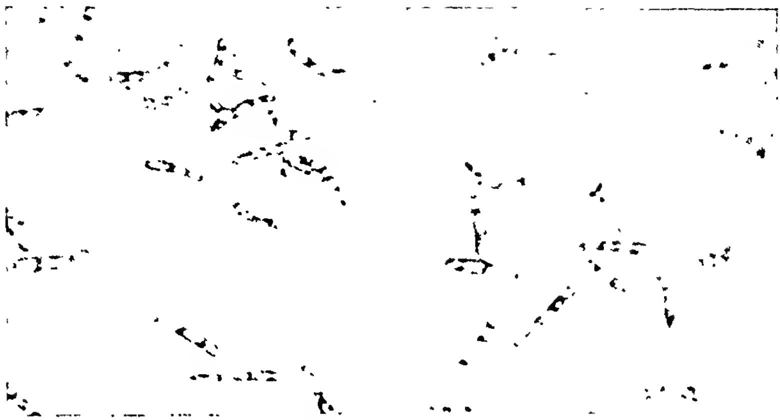

FIG 1

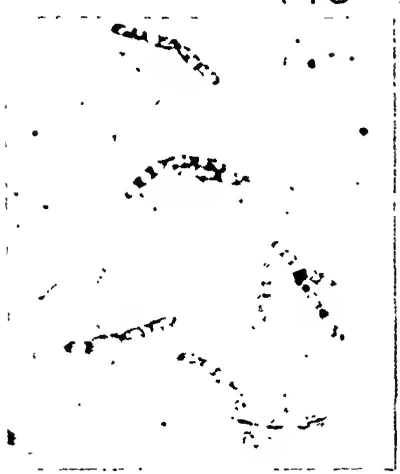

FIG 2

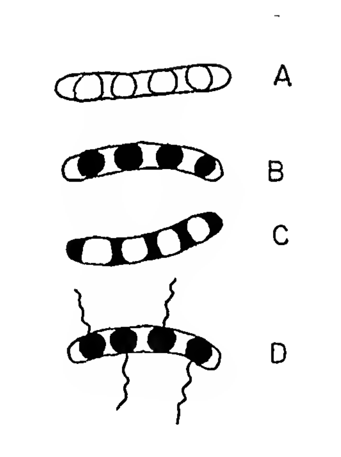

FIG 3

(I. M. Lewis: Cell Inclusions and Life Cycle of Rhizobia)

A QUANTITATIVE METHOD OF DETERMINING THE LETHAL EFFECT OF ULTRAVIOLET LIGHT ON BACTERIA SUSPENDED IN AIR

D. GORDON SHARP

Departments of Surgery and Bacteriology, Duke University School of Medicine and Duke Hospital, and the Department of Physics, Duke University, Durham, North Carolina

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INTRODUCTION

The use of ultraviolet rays to reduce the number of infections from air-borne bacteria in the operating room (Hart, 1936, 1937a and b; Hart and Gardner, 1937) has stimulated interest in the development of a technique to measure quantitatively the minimal dose of a particular kind of radiation necessary to sterilize air containing pathogenic bacteria. A great amount of work has been done on bacteria using various kinds of rays and exposing the organisms on solid media. The work of Gates (1929) on *Staphylococcus aureus* and other organisms gives percentages killed as a function of the energy used. He plotted a series of these curves for different wave lengths of light and found that 2660 Å was the most efficient wave length, 88 ergs per square millimeter being necessary for 50-per cent killing of *Staphylococcus aureus* on plates. In these experiments the bacteria were sprayed on a solid medium, exposed to radiation and the plates incubated to determine growth. This technique brought up several questions concerning the mechanism of the killing reaction. That bacterial death is a secondary effect brought about by photochemical changes in the media has long been discussed and not entirely agreed upon at present. Furthermore, aside from the possible changes in hydrogen peroxide concentration, pH, etc., that might be contributing factors there is another variable of a purely mechanical nature. No matter how uni-

formly one sprays a culture of bacteria on an agar surface there is a chance of screening one organism by another. This effect might be of considerable magnitude where soft, non-penetrating rays are used on organisms that normally develop clusters in culture media.

Some of these difficulties have been avoided in the method to be described in which the rays exert their effect on bacteria suspended in the air. Death of all exposed organisms has been taken as the end point.

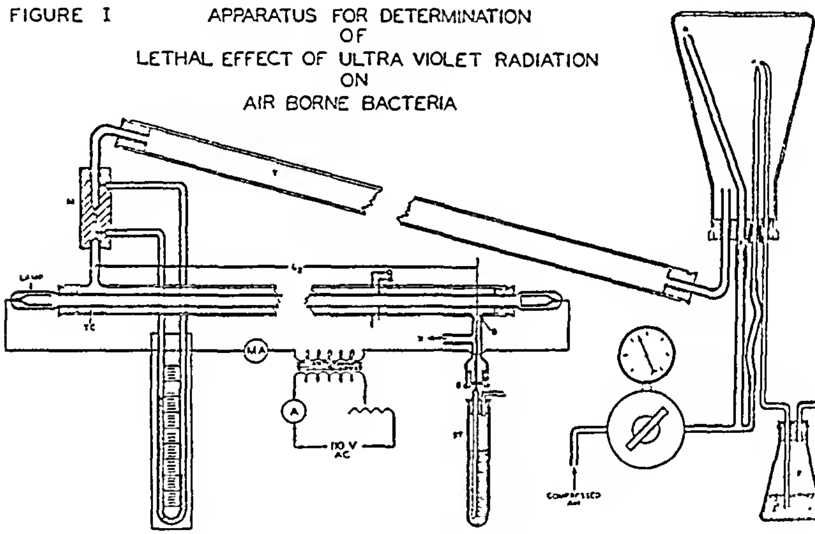

FIG. 1. DESCRIPTION OF APPARATUS

APPARATUS

General

The principle involved in this apparatus (fig. 1) is to drive a mixture of air and viable bacteria at constant rate through a test chamber where it is treated with a known amount of radiant energy. At the point where this mixture leaves the test chamber a method of sampling is provided. An aliquot part of the treated air is bubbled continuously through sterile broth during the entire length of the experiment. The broth is then incubated under optimum growth conditions for the organism involved.

Control is accomplished by taking similar samples without the radiation.

Spray system

A broth culture of bacteria is diluted in sterile saline solution and placed in flask (F) (fig. 1), which is connected to the spray chamber. Compressed air is then admitted through the control valve shown and passes at high speed through the jets (j) drawing with it this saline suspension of organisms and atomizing it into the chamber. This is accomplished at the rate of only about 10 cc. per hour, the liquid jet being small to prevent large droplet formation.

Into the spray chamber is also fitted another tube (K) for admitting additional sterile air to make up the desired flow velocity and allow for adjustment. In this manner air can be mixed with the organism to be tested and both volume of mixture per unit time and number of bacteria per unit volume can be held constant or varied at will.

Lamp

The source of ultraviolet rays used in this work was made by Westinghouse Electric and Manufacturing Company and consists of a high transmission glass tube about 28 inches long by $\frac{1}{2}$ inch in diameter filled with a mixture of Argon and Neon with mercury and fitted with 50 milliampere cold electrodes. It operates on a small transformer from 110 volts A.C. mains and requires about 400 volts at 30 ma. At this rating it uses approximately 10 watts power and rises 5° or 6°C. above room temperature. Its output intensity is roughly proportional to the current input (fig. 2) and the spectral distribution may be seen in figure 3. From this figure it is seen that the only strong line in the bactericidal part of the spectrum is the 2537 Å line. Measurements have shown that 88 per cent of the total output of this lamp, as measured on a bismuth-silver vacuum thermopile with quartz window is in this single line. The remaining 12 per cent lies largely above 3000 Å or above the bactericidal range.

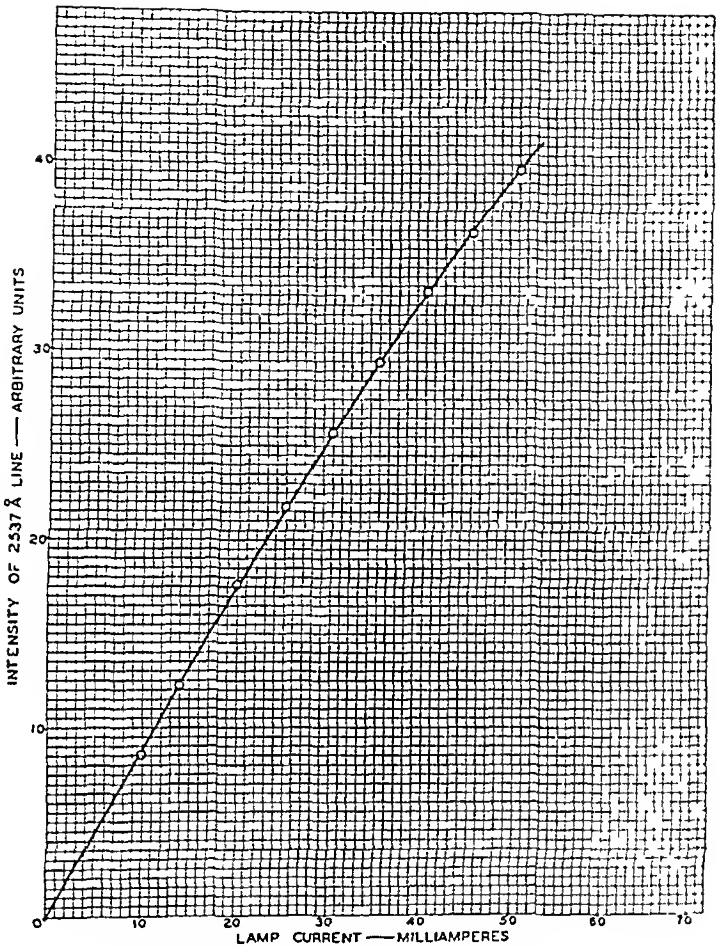

FIG 2 GRAPH SHOWING RELATIONSHIP OF OUTPUT INTENSITY TO CURRENT INPUT

FIG 3. SPECTROGRAM OF LAMP OUTPUT AT 27 MA CURRENT

Venturi meter

The stream of contaminated air is passed through a Venturi meter (M) to measure the rate of flow through the apparatus in cubic centimeters per second. This instrument indicates the rate of air flow as a function of the water level in a manometer and is thus a direct reading instrument.

Treatment chamber

After checking the velocity of flow the air is then passed through the treatment chamber ($T.C.$) which is a glass tube concentric with the ultraviolet lamp and forming a shell around it. The air passes between the lamp and this shell through a region where the intensity of the radiant flux can be accurately calculated. The product of this intensity by the length of time necessary to kill all the bacteria exposed is taken as a measure of the lethal dose.

Sampling tubes

In order to test the air issuing from the treatment chamber at (B) to determine sterility a tube of nutrient broth is used ($S.T.$). A $2\frac{1}{2}$ inch hypodermic needle (16 gage) is fastened to a glass capillary extending to the bottom of the tube. When this needle and tube with broth contents have been autoclaved it constitutes the sampling unit. Several of these are necessary in practice for a complete run.

The needle of the sampling tube is inserted through the serum bottle stopper (S) in the treatment chamber and a part of the treated air bubbles through the broth, the rest escaping through the side arm (X) which may be passed through an antiseptic solution when pathogenic organisms are used. After 48 hours incubation at 37.5°C. failure to cloud is taken as evidence of sterility of the tested air.

CALIBRATION

Lamp

A standard constant radiation lamp was obtained from the National Bureau of Standards and set up according to their

specifications to calibrate a bismuth-silver vacuum thermopile with quartz window. This thermopile was then used to measure the output of the ultraviolet lamp and 88 per cent of this value was taken as that which was active in the regions mentioned.

Venturi meter

This meter was of the common laboratory type calibrated with a gas meter checked on a National Bureau of Standards meter prover. It actuated a water manometer and gave a difference in water columns of about 6 cm. for an air velocity of the order of 100 cc. per second.

CALCULATIONS

Light intensity

The thermopile calibrated above was used to measure the output from 15 cm. of the lamp length at a point 40 cm. from the center of this length and perpendicular to it (fig. 4). Measurements could not be taken at a great distance from the lamp because of its low intensity. They could not be taken closer than a few centimeters because of the finite lamp size and consequent error. Thus it was necessary, for reasons to be discussed later, to calculate what the intensity would be at a point far from the lamp as a function of a reading taken at lesser distance. This value was then used to get the final result, the intensity in the treatment chamber (fig. 4).

If we choose a point, P_2, far from the lamp (fig. 4) the intensity at this point due to the whole length L_2 can be shown to bear the following relation to the intensity at point measured—P_1.

$$I_{P_2} = I_{P_1} \frac{S_1}{S_2} \sqrt{\frac{4S_1^2 + L_2^2}{4S_2^2 + L_2^2}} = I_{mh} \tag{1}$$

This value is known as the mean horizontal intensity and it has been shown (Kunerth, 1929) that the mean intensity I $m.s.$ over a sphere about the lamp center and of radius large with respect to L_2 is given by the following formula:

$$I_{ms} = \frac{\pi}{4} I_{mh} \tag{2}$$

It has been assumed that Lambert's Cosine Law is valid for this source.

Integrating over the whole sphere we get the total radiant flux from the lamp at the current measured. For any other current the corresponding output can be obtained from figure 2 which shows, in arbitrary units, the variation of output radiant energy with lamp current. This flux is then divided by the area of the treatment chamber to give the treatment intensity *IT*.

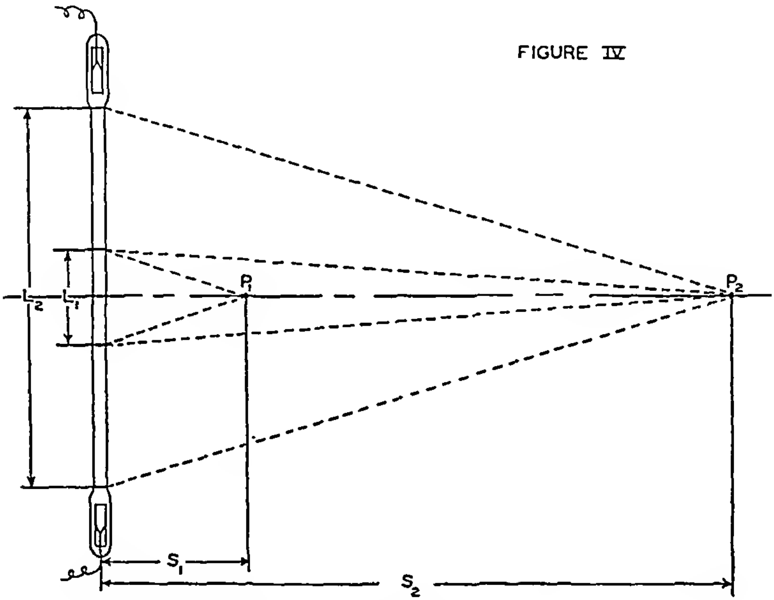

FIG. 4. DIAGRAM ILLUSTRATING THE BASIS OF CALCULATION OF INTENSITY
See text for explanation

Let $I_{P_1L_1}$ be intensity at P_1 due to length L_1 of lamp (calibration).

Let $I_{P_2L_2}$ be intensity at P_2 far from lamp $S_2 \gg L_2$.

$$I_{P_2L_2} = \frac{L_2}{L_1}\frac{S_1}{S_2}\sqrt{\frac{4S_1^2 + L_1^2}{4S_2^2 + L_2^2}} \times I_{P_1L_1}$$

$$I_T = \frac{\frac{\pi}{4} I_{P_1L_1} \frac{L_2}{L_1}\frac{S_1}{S_2}\sqrt{\frac{4S_1^2 + L_1^2}{4S_2^2 + L_2^2}} \times 4\pi S_2^2}{\pi D L_2}$$

$$I_T = \frac{\pi S_1 S_2 I_{P_1L_1}}{L_1 D}\sqrt{\frac{4S_1^2 + L_1^2}{4S_2^2 + L_2^2}}$$

but $S_2 \gg L_2$

so
$$I_T = \frac{\pi S_1 S_2 I_{P_1 L_1}}{2 L_1 D S_2} \sqrt{4S_1^2 + L_1^2}$$

$$I_T = \frac{\pi S_1 \sqrt{4S_1^2 + L_1^2}}{2 L_1 D} I_{P_1 L_1} \tag{3}$$

It should be noted here that in the calculations which follow, the diameter (D) of the treatment chamber has been taken to the outside of the chamber in order to give the weakest part of the field.

Exposure time

It has been assumed that the bacteria pass through the chamber at the same rate as the air. Although it is probable that they do not have the same rate of flow, at least their velocities will be grouped about this value and it has been taken as a first approximation. In order to calculate the exposure time (T) it is necessary to divide the volume of the treatment chamber by the air flow in cubic centimeters per second.

$$T = \frac{\pi L_2 (D^2 - d^2)}{4 \times \text{cc./sec.}} \tag{4}$$

Energy

The energy per unit area, to which a bacterium has been exposed, will be given by the product of the treatment intensity I_T and the treatment time T.

EXPERIMENTAL

A strain of *Staphylococcus albus* was isolated from the air and cultivated on beef-extract agar slants. Twenty-four hour broth cultures (37.5°C.) inoculated from these slants were used for each experiment and diluted 1 to 50 in sterile 0.85 per cent NaCl solution before placing in the flask (F).

Table 1 gives the results of three tests taken on different days. All tubes were incubated 48 hours at 37.5°C. and examined microscopically as well as macroscopically to determine sterility.

As an added check the following test was made. The lamp

was operated brightly enough to kill all the organisms coming through as evidenced by sterility in the exposed sampling tube. At the same time the air issuing from (X) was directed against a

TABLE 1

Venturi meter water column	6.6	cm.
Calculated air flow	113.0	cc./sec.
Calculated exposure time	1.06	sec.
Length of run per sample tube	7.0	min.
Volume air treated per sample	47.5	liters*

1. LAMP CURRENT	24-HOUR INCUBATION	48-HOUR INCUBATION
ma.		
00.0	Cloudy	Cloudy
50.0	Clear	Clear
33.4	Clear	Clear
27.3	Clear	Clear
21.2	Cloudy	Very cloudy
15.0	Cloudy	Very cloudy
10.0	Cloudy	Very cloudy
00.0	Not taken	Cloudy
29.3	Not taken	Clear
26.6	Not taken	Cloudy
24.2	Not taken	Cloudy
22.0	Not taken	Cloudy
19.8	Not taken	Cloudy
00.0	Cloudy	Cloudy
29.3	Clear	Clear
26.6	Clear	Clear
24.2	Clear	Clear
22.2	Cloudy	Cloudy
19.8	Cloudy	Cloudy

Maximum variation 21.2 ma. to 29.3 ma.

Output of lamp in 2537 Å line measured 40 cm. distance from center of a 15 cm. exposed length operating at 50 ma.—29.51 mw. per square centimeter.

* Twenty-five per cent of this air bubbled through the broth of the sampling tube (*ST*).

drop of sterile saline solution on a microscope slide. After thirty minutes operation the slide was stained and found to contain well-scattered gram-positive cocci in large numbers.

Taking 25.0 ma. lamp current (table 1) as the mean value from

the above data, using the lamp calibration cited with the data, and applying the equations developed under "calculations" it is seen that these organisms have been exposed to a minimum of 26,200 ergs per square centimeter of area. This energy was chiefly 2537 Å radiation and is only slightly lower than the value of 30,000 ergs per square centimeter obtained by Gates (1929) with *Staphylococcus aureus* on plates for the same wave length.

DISCUSSION

The radiant intensity is, of course, not constant throughout the treatment chamber. An effort has been made to minimize this variation by keeping the spacing between the walls as small as possible. However, as this intensity is naturally higher near the lamp at (d) than it is at (D), the outside wall, the latter value has been used in the calculations of (I_T). This, together with the exposure time, should give the minimum lethal dose of a given wave length for a given species of bacteria.

As has been pointed out, the lamp operates on low power and so the glass surface does not rise more that 5° or 6°C. above room temperature. Direct killing by heat should not then be an important factor in the action of this lamp.

In this method of measurement the bacteria are observed and treated under similar conditions of air suspension to those experienced in the operating room. This should make the data useful in any such air problem and render it free of certain common drawbacks of the usual method of plate exposure. There is no complication of the results by such issues as photochemical alteration of the supporting media.

Another phase of bacterial resistance to light and one which some authors (Wells and Wells, 1936) have considered of some importance is presented by the characteristic grouping of organisms. In the case of *Staphylococcus albus* the characteristic grape-like clusters which this organism forms have been said to contribute greatly to its resistance to ultraviolet rays of low penetrating power. In the present method of observation it seems that there are few clusters that are not broken up by the fine, high speed spray jets. The glass slide exposed to the

exhaust air caught only well scattered organisms alone and in pairs.

SUMMARY

Features of the experimental method:

1. Broth cultures of bacteria are atomized into the air.
2. The mixture of bacteria and air is passed through a tube where it is exposed to ultraviolet light.
3. The air is then passed through nutrient broth which can be plated out and count made or merely incubated and examined for growth.
4. Measurements are made of:
   (a) Lamp current—to calculate treatment intensity (I_T).
   (b) Air velocity—to calculate exposure time (T).
5. Energy received per unit area by the bacteria is given by the product of $I_T \times T$ = Energy.

Preliminary results using this method on *Staphylococcus albus* have shown the energy necessary to kill all of the organisms was approximately 26,200 ergs per square centimeter. This is in good agreement with the current work using the method of plate exposure.

REFERENCES

Gates, F. L. 1929 A study of the bactericidal action of ultraviolet light. Jour. Gen. Physiol., 13, 231–260.

Hart, Deryl 1936 Sterilization of the air in the operating room by special bactericidal radiant energy. Jour. Thoracic Surg., 6, 45–81.

Hart, Deryl 1937a Operating room infections. Arch. Surg., 34, 874–896.

Hart, Deryl 1937b Sterilization of the air in the operating room by bactericidal radiant energy. Surgery, 1, 770–784.

Hart, Deryl and Gardner, C. E. 1937 Sterilization of the air in the operative region with bactericidal radiant energy. Trans. Southern Surg. Assoc., 49, 376–402.

Kunerth, W. 1929 A Textbook on Illumination, pp. 67–68. John Wiley & Sons, New York, New York.

Wells, W. F., and Wells, W. M. 1936 Air borne infection. Jour. Amer. Med. Assoc., 107, 1805–1809.

RESPIRATORY ENZYME SYSTEMS IN SYMBIOTIC NITROGEN FIXATION

I. The "Resting Cell" Technique as a Method for Study of Bacterial Metabolism

P. W. WILSON[1]

Biochemical Laboratory, University of Cambridge, England, and Department of Agricultural Bacteriology, University of Wisconsin

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Studies on the biochemistry of symbiotic nitrogen fixation (Wilson and Fred, 1937) indicate that oxidation reactions play a significant, though probably indirect, rôle in this process. A survey of the properties of the respiratory enzymes in species of the root-nodule bacteria (*Rhizobium sp.*), both when grown alone and with the proper host plant, appears necessary for progress on the problem of the mechanism of the reaction. Previous studies of the respiratory activities of the root-nodule bacteria (Georgi and Wilson, 1933; Neal and Walker, 1935, 1936; Thorne and Walker, 1935; Thorne, Neal and Walker, 1936; Walker, Anderson and Brown, 1934, a, b) were made primarily with growing cultures. Interpretation of the data is difficult since effects on growth are not readily differentiated from effects on respiration.

The "resting cell" techniques eliminate many of the objections associated with studies made on growing cultures (Konishi and Tsuge, 1934). Resting cells, or more accurately, non-proliferating cells are washed suspensions of bacteria which are used as a source of enzymes for studies on bacterial metabolism. The enzyme is not separated from the cell but is "isolated" through use of specific substrates, inhibitors and other means. There is evidence (Quastel, 1928, 1932; Stephenson, 1930) that certain

[1] John Simon Guggenheim Memorial Foundation Fellow 1936. The author is grateful to the Foundation for its grant to conduct research in consultation with European authorities on the problem of nitrogen fixation by bacteria.

enzymes concerned with energy-liberating reactions consist primarily of definite patterns of molecules in the cell or on its surface. The activity of such enzymes is a function of the orientation of these molecules in the surface. Separation of this type of enzyme from the cell by the usual methods would be a self-defeating procedure, as, obviously, dissolution of the cell would result in destruction of the enzyme. Resting cells are of particular value for the study of reactions catalyzed by such "structure bound," enzymes.

Some other advantages of the use of resting cells may be cited:

1. Respiratory activities are separable to a considerable extent from those associated with growth. Since no source of nitrogen is supplied, proliferation of the cells is at a minimum, and the reactions are greatly simplified. With growing cultures, separation of growth and respiration responses is difficult and frequently impossible.

2. The kinetics of a reaction can be readily followed. Consideration of the *rates of reaction* rather than *total reaction* is frequently advantageous and usually desirable for accurate interpretation of results (Werkman, 1927; Burk, 1934).

3. Single reactions may be studied rather than the combined effects of several. If formate is the sole substrate present, then resulting changes arise from decomposition of formate. This "isolation" of specific reactions is of great value for the investigation of possible intermediates in the decomposition of a given substrate. Such isolation is usually difficult with media which allow growth.

4. Closely related reactions often may be differentiated by use of specific inhibitors or by determination of the characteristic properties of the enzyme systems concerned with the reactions.

5. Results are referred to a standard which allows the data from one experiment to be directly compared with those from another. For this purpose the constant, Q_{O_2}, defined as the cubic millimeters of oxygen consumed per hour per milligram dry weight of organism is usually employed. As this constant represents a *rate per unit weight*, the original data are corrected for differences in the mass of organisms in various suspensions.

Similar constants can be calculated for uptake or disappearance of substances other than oxygen. If growing cultures are used, it is difficult to correct for differences in number or mass of organisms since the values change with time.

6. The short-time nature of the experiments eliminates effects arising from differences in development of cultures. Also, once the technique has been developed, many experiments can be made in a single day, as the actual measurements usually require an hour or less.

The cited advantages are exemplified by the outstanding investigations on bacterial metabolism of the Cambridge University group. Solution of many of the problems attacked by this group of workers would have been delayed if the conventional methods of bacteriology had been employed. Among these are: the resolution of the hydrogen enzymes of *Escherichia coli* (Stephenson and Stickland, 1931; Stephenson, 1937; Woods, 1936); the manner in which some strict anaerobes secure their energy (Stickland, 1934); adaptive *versus* constitutive enzymes (Stephenson and Yudkin, 1936; Stephenson and Gale, 1937; Quastel, 1937).

Although for the past decade English investigators have employed the resting cell techniques for study of bacterial metabolism, similar investigations in America are rather uncommon. Werkman and his collaborators (Stone and Werkman, 1937; Wood, Stone and Werkman, 1937) used non-proliferating cells for studies of intermediates in bacterial fermentations. Others employed such suspensions for investigations of the dehydrogenases of bacteria using the methylene-blue technique (Kendall and Ishikawa, 1929). However, detailed unvestigations of specific enzyme systems, through use of the micro-respirometer together with the methylene-blue technique and isolation of chemical compounds have been quite neglected. Part of the delay, no doubt, arises from the slow adoption of the micro-respirometer for metabolic studies. The future will probably witness increasing replacement of the traditional methods for study of bacterial metabolism by techniques using resting cells.

This paper suggests the type of problem which may be advantageously undertaken through use of the resting cell techniques

and details the preliminary survey that must be made when the metabolism of a new group of organisms is investigated. The actual data will be of chief interest to those studying the specific organism used, but the same general procedures are applicable to a study of almost any group of microörganisms.

METHODS

The measurement of oxygen uptake with the Warburg or the Barcroft respirometer has been described (Dixon, 1934). The papers of the Cambridge workers already cited should be consulted for details of the general procedures for preparation of the suspensions of bacteria and for determination of methylene-blue reduction. *Dry weight* was estimated by drying 2 cc. of the suspension at 100°C. and deducting the weight of the salts in the suspending solution. *Total nitrogen* was determined by the modified Pregl micro-method or by the semi-micro method of Umbreit and Bond (1936). *Hydrogen-ion* estimations were made with the glass electrode. Wisconsin strain 209 of *Rhizobium trifolii* was used as the test organism. Purified chemicals were used in the media and special chemicals (Kahlbaum or Eastman) as the substrates. Both the Barcroft and the Warburg types of micro-respirometer were employed.

PREPARATION OF SUSPENSIONS

Before actual measurements of the respiratory activities of an organism are undertaken, methods for preparing a suspension with satisfactory properties must be developed. These include the following points: (*a*) the organisms should be readily separable fron the suspending or washing solutions; (*b*) the cells should possess a high respiration rate, otherwise the quantity necessary for estimation of oxygen uptake may introduce complicating factors; (*c*) the *endogenous* metabolism, that is, the respiration of the organisms alone, should not be excessive; (*d*) the rate of respiration should be directly proportional to the number of viable organisms present; (*e*) the activity of the suspension should be stable, otherwise it is necessary to prepare a fresh suspension for each determination.

The first attempts to prepare a satisfactory suspension of *R. trifolii* failed. The organisms were grown on medium 79, a yeast-extract mineral-salts mannitol agar substrate (Fred, Baldwin and McCoy, 1932), washed from the agar surface with Allison's solution[2] (Allison and Hoover, 1934), and a suspension prepared by the usual technique. The rate of respiration on glucose of this suspension was very low, the Q_{O_2} being less than 10. Most bacteria have a Q_{O_2} on glucose of 20 to 50, and a few species may exceed 100. Recovery of the organisms from the suspending solution was very poor even with prolonged centrifuging. Moreover, the endogenous respiration of the cells was about 60 per cent of that on glucose. Such preparations are almost useless for study of oxygen uptake on different sources of carbon since most of the respiration arises from cellular constituents and not added substrate.

The cause of the difficulties was the production by the bacteria of a gum which is not readily removed from the cell. This gum gave the organisms colloidal properties which interfered with their separation from liquids, it lowered the Q_{O_2} of the cells since their dry weight included adhering gum, and it provided a substrate for the organisms which resulted in a high "endogenous" respiration. The cells were very low in percentage of nitrogen; if the rates of respiration were calculated ôn basis of nitrogen content, the values were comparable with those for other microorganisms.

Apparently the difficulties would be overcome if the nitrogen content of the cells could be raised. This was accomplished by growing them on a yeast-extract mineral-salts agar without added carbohydrate. On this medium the organisms grew readily, produced little gum, were easily separated from suspending and washing media, were high in percentage nitrogen and possessed satisfactory respiratory activity.

Both total crop of organisms and percentage of nitrogen in the cells increased as the quantity of yeast extract in the medium was raised from 0.25 to 1.0 per cent, but further increases had

[2] Preliminary experiments indicated that this solution of balanced salts gave better suspensions of *R. trifolii* than did the customary normal saline solution.

little effect. In subsequent experiments the medium consisted of one-per-cent yeast extract, the salts of medium 79 and 2.5 per cent agar. The agar was increased from 1.5 to 2.5 per cent as this gave the medium physical properties which aided in the removal of the cells from the surface.

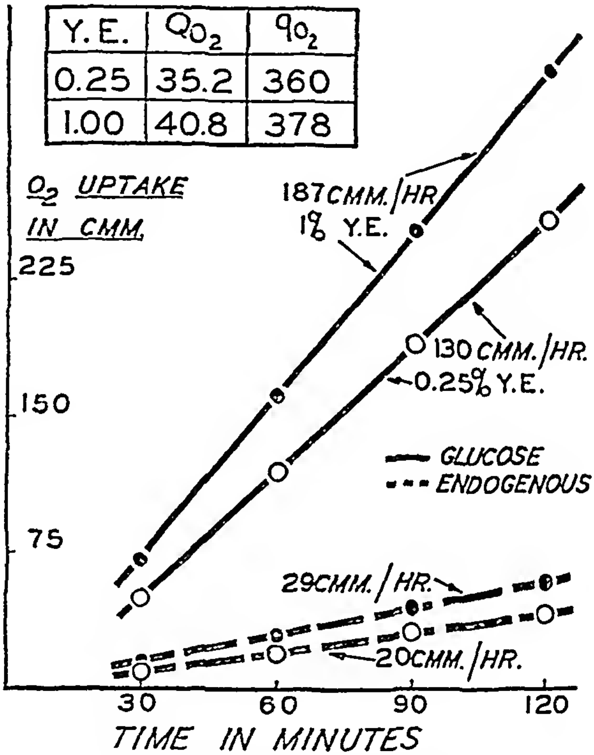


FIG. 1. EFFECT OF CONCENTRATION OF YEAST EXTRACT IN MEDIUM ON RESPIRATION OF R. TRIFOLII

Dry weight of suspension grown on 0.25 per cent yeast extract was 3.7 mgm. per cubic centimeter; nitrogen content, 0.362 mgm. per cubic centimeter. Dry weight of suspension grown on 1.0 per cent yeast extract, 4.6 mgm. per cubic centimeter; nitrogen content, 0.495 mgm. per cubic centimeter.

Respiration data from two suspensions are presented in figure 1 which illustrates the method for calculating the Q_{O_2}. The oxygen uptake of a bacterial suspension is plotted against time, and the slope of the resulting line is determined. Theoretically, it requires only two points to determine this slope, but in practice

at least four points are taken in order to make sure that the rate of respiration is constant. The slope of this line in cubic millimeters per hour divided by the *dry weight* in milligrams of the organisms in 1 cc. of suspension gives the Q_{O_2}.

Values of the Q_{O_2} increased with concentration of yeast extract in the medium, probably because of a decreased gum formation. If the *nitrogen content* of the organism were used to calculate a constant, q_{O_2}, defined as the oxygen uptake in cubic millimeters per hour per milligram N in the cells, the values obtained were essentially independent of gum production. Because of the more stable character of the constant based on nitrogen content, this datum was used rather than the traditional Q_{O_2}. As can be seen in the figure, the endogenous respiration of the organisms grown in the absence of added carbohydrate constitutes only 15 to 20 per cent of the respiration on glucose.

During endogenous respiration, NH_3 was liberated, but none was formed by cells given a source of carbon. This result suggests that endogenous respiration represents, at least in part, oxidative deamination of cellular amino acids and that this type of oxidation does not occur with cells given a source of readily available carbon (protein-sparing action of carbohydrates). For this reason the oxygen uptake of cells in the absence of substrate was not subtracted from that in the presence of substrate before calculation of the q_{O_2}.

As shown in table 1, the addition of sucrose to the basic yeast-extract medium caused the percentage of nitrogen in the cells and the Q_{O_2} to decrease, and the endogenous respiration relative to that with glucose to increase. Also, as addition of sucrose caused a greater production of gum, the efficiency of separating the cells from the suspending liquid was decreased. To the eye, the largest crop was formed in the presence of the higher concentrations of sucrose, but the greatest crop recovered on centrifuging and washing was obtained from the medium with the lowest level of carbohydrate.

Since all the effects of adding sucrose are undesirable insofar as the respiratory activity of the resulting suspensions are concerned, no carbohydrate was used in the standard medium adopted. In

general, growth of the organism in the absence of added carbohydrate should be advantageous since respiration studies on different sources of carbon would not be complicated through previous "adaptation" of the bacteria to a specific carbohydrate. If a preliminary adaptation is wanted, as much as 0.1 per cent carbohydrate can be added to the medium used to grow *R. trifolii* with little loss in the desirable properties of the suspension.

The data in table 2 show that the optimum time for harvest is about 48 hours since at this time the q_{O_2} has decreased only

TABLE 1

Effect of addition of sucrose to yeast extract medium on properties of suspensions of R. trifolii

SUCROSE ADDED	CROP YIELD PER CUBIC CENTIMETER			RATE OF RESPIRATION	Q_{O_2}	q_{O_2}	ENDOGENOUS RESPIRATION
	Dry weight	Total N	Per cent N				
per cent	*mgm.*	*mgm.*		*cmm. per hr.*			*per cent**
0.05	3.50, 3.45, 4.00	0.348, 0.348, 0.318	9.26	139, 138	37.9	410	15.3
0.10	4.00, 3.30, 3.25	0.266, 0.259, 0.259	7.42	116, 113	32.5	438	16.6
0.25	3.40, 3.20, 3.60	0.208, 0.259, 0.222	6.76	100, 96	28.8	426	21.0

* Per cent of total respiration on glucose.

slightly from the maximum obtained at 24 hours, and the total crop of cells has almost reached the maximum. The percentage nitrogen in the cells increased, and the endogenous respiration relative to that on glucose decreased with age of the culture. Both observations suggest that part of any carbohydrate-like material present in the yeast extract is converted into gum early in the development of the organism, and with the exhaustion of this readily available source of carbohydrate, the gum is utilized (cf. Georgi and Wilson, 1933).

As a result of these preliminary investigations, the following

method was adopted for preparation of suspensions of the organisms:

Technique for preparation of suspension. Stock cultures of *R. trifolii* are kept on a medium consisting of the mineral salts of Medium 79 plus 0.5 per cent Difco yeast extract (9 per cent nitrogen) and 1.5 per cent agar. The growth of a 48-hour-old

TABLE 2

Effect of age of culture on properties of suspensions of R. trifolii

AGE OF CULTURE	YIELD OF CROP				RATE OF RESPIRATION	Q_{O_2}	q_{O_2}	ENDOGENOUS RESPIRATION
	D.W. per cc.	N per cc.	Per cent N	Total N* in crop				
hours	*mgm.*	*mgm.*		*mgm.*	*cmm. per hour*			*per cent*†
24		0.208 0.185 0.178		0.190	92 92 94		485	21.3
48	5.80 5.70 5.65	0.490 0.490 0.460	8.41	2.41	203 203 209	35.8	428	18.6
72	6.20 5.90	0.630 0.645 0.636	10.51	3.18	189 189 187	31.1	295	16.4
96	4.10 4.05	0.437 0.445 0.445	10.82	2.95	86 85 80	20.6	190	16.2

* Per bottle basis:
24 hours—organisms from 6 bottles made up to 6 cc.
48 hours—organisms from 4 bottles made up to 20 cc.
72 hours—organisms from 4 bottles made up to 20 cc.
96 hours—organisms from 3 bottles made up to 20 cc.

† Per cent of total on glucose.

slant is washed with 10 cc. of sterile Allison's solution into a sterile flask provided with sterile beads. Two cubic centimeters of a uniform suspension from several slants is spread over 100 cc. of a one-per-cent yeast-extract mineral-salts 2.5-per-cent-agar medium contained in a liter Roux bottle. A litmus milk tube is also inoculated with one drop of the suspension in order to test

the purity of the culture (Fred, Baldwin and McCoy, 1932). After incubation at 28° to 30°C. for 48 hours, the organisms are harvested. Ten to 15 cc. of sterile Allison's solution are added to each bottle, and the organisms transferred with the aid of a sterile glass rod into a sterile bottle containing beads. Organisms from replicate Roux bottles are combined and after thorough mixing are filtered through sterile glass wool to remove clumps. They are then centrifuged for 20 minutes at 3000 r.p.m., and after removal of the supernatant liquid are washed twice with sterile Allison's solution. The washed organisms are then suspended using about 10 cc. of sterile Allison's solution for each Roux bottle in the harvest. The suspension is kept at 3°C. in a 38 by 200 mm. pyrex test tube provided with means for aeration with aseptic precautions. Before use, the culture is aerated for 15 minutes at 0°C. One cubic centimeter of this suspension which contains 2 to 3 mgm. dry weight (about 0.25 mgm. N) is used for measurement of oxygen uptake or for reduction of methylene blue.

TESTS ON THE SUSPENSIONS

A desirable property of suspensions is that the organisms are in a state of maximum activity. Suspensions prepared by the technique just described met this requirement as the cells were in the log phase of development. If combined nitrogen and a source of energy were supplied to the organisms, the rate of respiration became a logarithmic function of time within a few hours (figs. 2 and 3). Allison and Hoover (1934) have described a factor which stimulates the growth of rhizobia in synthetic media. They claim that it acts as a coenzyme for respiration and have called it "Coenzyme R." Thorne and Walker (1936a, b) dispute that the factor is essential for growth or respiration and propose that the term used by Allison and Hoover be discarded in favor of "accessory growth substances." Since yeast extract is an excellent source of the accessory substance, the suspensions of *R. trifolii* used should be well supplied with the factor. That this view is correct was demonstrated in these growth experiments by adding to certain of the suspensions 100 p.p.m. of "Coenzyme R" prepared from *Azotobacter* (Hoover

and Allison, 1935). As can be seen in figures 2 and 3, such addition was without appreciable effect on the rate of growth, as

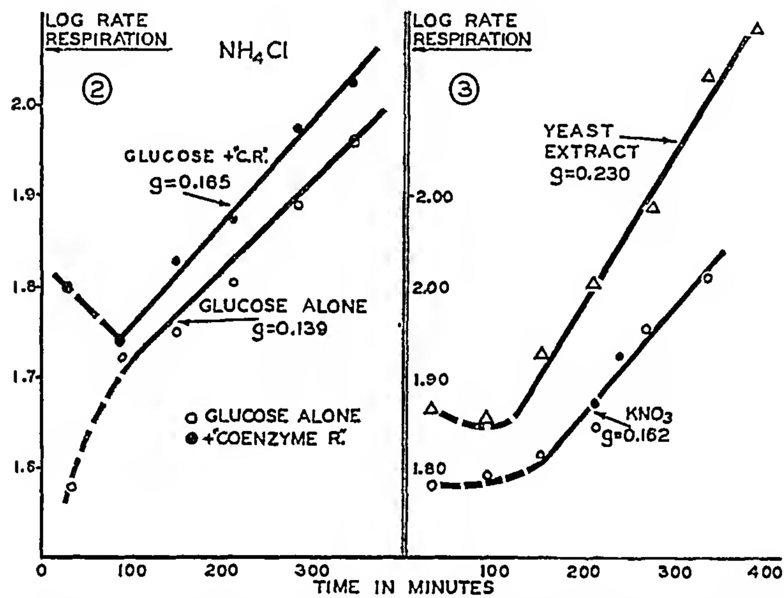


FIG. 2. EFFECT OF ADDITION OF NH_4Cl AND ACCESSORY GROWTH SUBSTANCES ("COENZYME R") ON RESPIRATION OF SUSPENSIONS OF R. TRIFOLII

FIG. 3. SAME AS FIGURE 2 WITH YEAST EXTRACT AND KNO_3 AS SOURCES OF NITROGEN

measured by increase in respiration. The values of the specific constant,

$$g = \frac{2.303}{t_2 - t_1} \log \frac{\text{respiration rate at } t_2}{\text{respiration rate at } t_1}$$

in which t refers to time, are probably identical within experimental error. That the respiration of these organisms is independent of added accessory substances ("Coenzyme R") was verified by adding 10 to 200 p.p.m. of the preparation to resting cells; no effect on the q_{O_2} could be detected.

Respiration of the organisms in these suspensions is directly proportional to the mass of cells. As shown in table 3, dilution of the suspensions as much as four-fold did not affect the q_{O_2}.

Suspensions may accordingly be directly compared without adjusting to some standard density of organisms. The data in the second part of table 3 show that the respiratory system of the cells is quite stable. The q_{O_2} on glucose of a suspension remains constant for at least 132 hours if kept in a refrigerator (3°C.). Such a property is very convenient, as a stock suspension can be used for tests made over a period of several days.

TABLE 3

Effect of dilution and storage of suspensions on rate of respiration (q_{O_2})

TREATMENT	SUSPENSION 2	SUSPENSION 3	SUSPENSION 4	SUSPENSION 5
		Effect of dilution*		
cc.				
2.0	123			
1.0	132	250	354	
0.5		230		
0.25		250	353	
		Effect of storage†		
hours				
0	127	215		411
12			354	
24	133	224		409
48				364
60		243	378	
84			353	
108			372	
132			368	

* Volume of suspension taken for measurement of q_{O_2}.
† Suspensions kept in ice-box at 3°C.

EFFECT OF ENVIRONMENTAL FACTORS

Once the chief factors concerned with the preparation of a satisfactory suspension have been established, the next step is to determine the proper technique for making the respiration measurements.

Effect of concentration of phosphate. In a typical experiment the following values for the q_{O_2} were obtained with mixed mono- and di-potassium phosphate buffers of different molality: M/3.33,

294; M/10, 387; M/30, 470; and M/90, 487. These data indicate that a definite decrease in the q_{O_2} occurs even with a buffer of phosphate molality as low as M/10. Because of this sensitivity of the organisms to high phosphate concentrations, and as lower

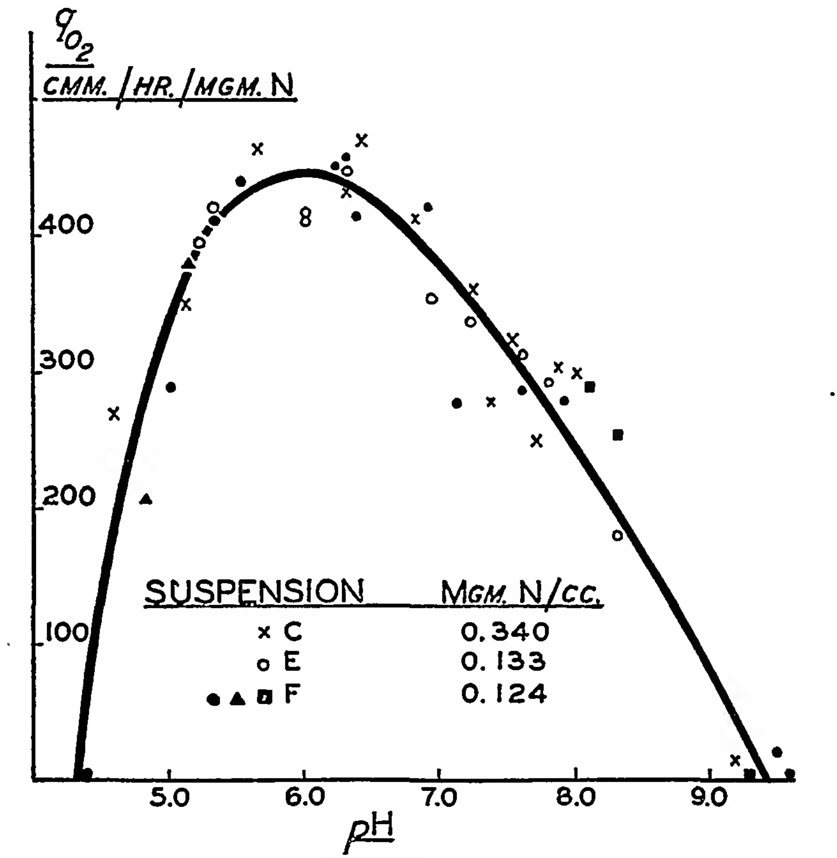


FIG. 4. RATE OF RESPIRATION OF R. TRIFOLII AS A FUNCTION OF pH
Buffer: × ○ ● KH_2PO_4-K_2HPO_4
▲ KH_2PO_4-KHphthalate
■ K_2HPO_4-borate

concentrations did not buffer sufficiently, all measurements were made in a solution of M/30 phosphates.

Effect of reaction. The pH function of the respiration of *R. trifolii* is shown in figure 4 obtained with three suspensions

whose density of cells, as measured by the nitrogen content, varied three-fold. The q_{O_2} values were so constant for the different suspensions that it was possible to plot in one figure all the data without adjustment.

In the region of the neutral point little change in the pH of the suspension plus buffer and substrate occurred during a test. In the extreme acid and alkaline ranges, however, the initial pH differed from the final by as much as 0.4 pH units, doubtlessly due to the low buffering capacity of the mixed phosphates in these regions. This change during a measurement necessitated use of the average value of the pH; hence, these points may not be too accurate. Addition of potassium acid phthalate or sodium borate to the phosphate buffer was shown to be without influence on the respiration. Accordingly, one test was made in which a KH_2PO_4 potassium-acid-phthalate buffer was used in the acid range and K_2HPO_4 borate was used in the alkaline. These buffers were quite stable, and as can be seen from the figure, the respiration values checked those obtained with the mixed phosphates which were open to suspicion.

The optimum pH for respiration of *R. trifolii* is in the range of pH 5.1 to 7.0; there is no well-defined maximum. On either side of the maximum range the rate of respiration decreases rapidly with limits of approximately pH 4.0 and of 9.0. Thorne and Walker (1935) found that the optimum pH for *growth*, as measured by increase in respiration, of *R. meliloti* (alfalfa group) and *R. japonicum* (soybean group) is 7.0. The rate of growth decreased rapidly on either side of the optimum to the limits for growth of pH 4.5 and 9.5. The data of Virtanen and v. Hausen (1931) indicate that the pH optimum for growth of *R. trifolii* on nutrient gelatin extends from 6.5 to 7.5 and that the optimum for fixation of atmospheric nitrogen by nodulated red clover plants is from 6.5 to 7.0 with limits at pH 4.0 and 8.5. Apparently, the optimum reaction for respiration by resting cells of *R. trifolii* is somewhat more acid than the previously observed values for growth of the various *Rhizobium* species. The pH-respiration function of the free-living nitrogen fixer, *Azotobacter*, exhibits a maximum at 7.2 to 7.5 (Burk, Lineweaver and Horner, 1934).

Effect of temperature. The optimum temperature for growth of rhizobia is 28° to 30°C. according to a number of investigators (Fred, Baldwin and McCoy, 1932). As shown in figure 5, however, the optimum for respiration is about 37.5°C. with little change in the rate of respiration throughout the range 35° to 39°C. Because of this rather unexpected difference in the temperature optima for growth and respiration, the effect of temperature on

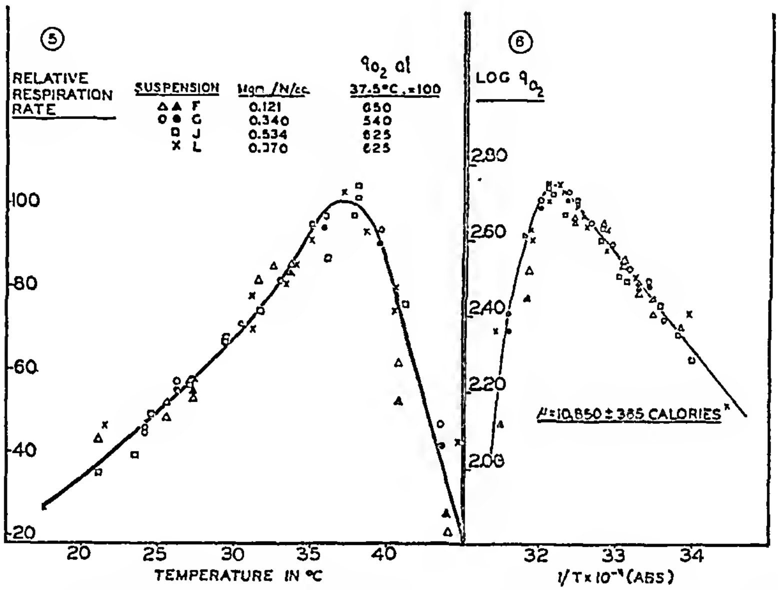


FIG. 5. INFLUENCE OF TEMPERATURE ON RATE OF RESPIRATION OF R. TRIFOLII ○ △ = fresh culture from ice-box. ● ▲ = culture used in previous determination.

FIG. 6. TEMPERATURE CHARACTERISTIC OF RESPIRATION OF R. TRIFOLII

respiration was determined with four different suspensions. As the q_{O_2} for the different suspensions varied to some extent, all were adjusted to a comparable basis by equating the q_{O_2} at 37.5°C. to 100 and calculating the relative values for the other temperatures used. At each temperature the rate of respiration was determined in duplicate. In some instances, at the conclusion of a measurement one of the duplicates was retained, and at the next temperature used, its respiration compared with that

of a fresh culture from the stock suspension kept in the refrigerator. No consistent differences between such duplicates, *i.e.*, fresh and "old," were observed.

When the logarithms of the q_{O_2} values at each temperature are plotted against the reciprocal of this temperature measured on the absolute scale, the points fall along a straight line. The slope of this line multiplied by 4.6 is equal to the Arrhenius temperature characteristic,

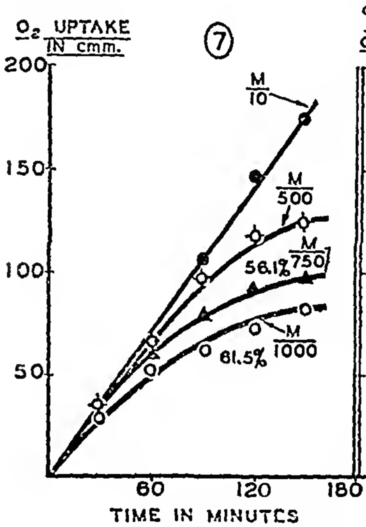
$$\mu = R[T_2T_1/(T_1 - T_2)] \log r_2/r_1$$

in which R is the gas constant, 1.99 cal.; r_1, the rate of respiration at the absolute temperature T_1; r_2, the rate of respiration at the absolute temperature T_2. The mean value of μ for the four suspensions is 10,850 ±365 cal. In figure 6 is shown the fit of the points to a line with a slope corresponding to this value of μ after the individual values for each suspension had been adjusted to a common basis. The observed μ is within experimental error of the value, 11,000 calories which Crozier (1924) found to be typical for a large number of biological oxidation reactions. The value of μ for *Azotobacter*, 19,330 ±165 cal., is considerably higher, and the optimum for this organism, 34.5° to 35°C., is definitely lower (Lineweaver, Burk and Horner, 1932).

Effect of concentration of substrate. As indicated in figure 8, the *initial rate of respiration* of glucose by *R. trifolii* is independent of the molality over a hundred-fold range, M/10 to M/1000. With concentrations less than M/100 the rate of respiration decreases with time. Under these circumstances, it is difficult to estimate accurately the *initial rate of respiration*; this probably accounts for the slightly lower value found for M/1000 glucose. When low concentrations of the substrate were used, the respiration practically ceased with an oxygen uptake corresponding to 60 per cent of the glucose present. A similar observation made by Cook and Stephenson (1928) for oxidation of glucose, lactate and acetate by *Escherichia coli* has been recently explained by Clifton (1937). Clifton showed that part of the acetate was *assimilated* by the cells, but if assimilation was prevented by addition of low concentrations of sodium azide or dinitrophenol,

oxidation proceeded to completion. It is likely that part of the glucose is incompletely oxidized to gum by the root nodule organisms.

Technique for making measurements. On the basis of these determinations of the optimum environmental conditions for respiration of *R. trifolii* with glucose as the substrate, the following standard procedure was adopted. To each Warburg flask is added 1 cc. of the suspension of organisms, 1 cc. of M/30



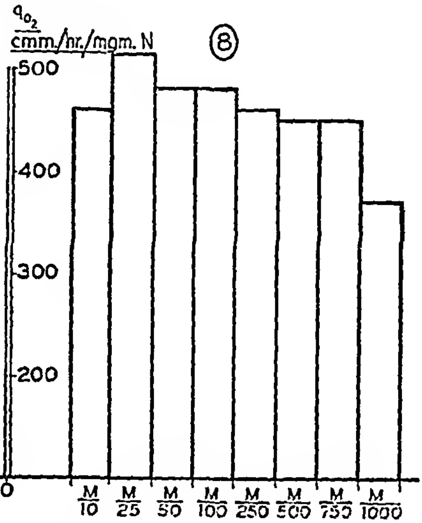


FIG. 7. CURVES OF OXYGEN UPTAKE BY R. TRIFOLII

One cubic centimeter used of each concentration of glucose indicated. Curves for concentrations to M/250 coincide with that shown for M/10. Curve for M/250 begins to drop away from this curve after two hours.

FIG. 8. EFFECT OF CONCENTRATION OF GLUCOSE ON THE INITIAL RATE OF RESPIRATION OF R. TRIFOLII

mixture of phosphates (pH 6.2) and 1 cc. of M/50 substrate. The temperature of the bath is maintained at 34°C. With temperatures near 40°C. a decrease in the rate of respiration with time was often observed. The rate of respiration is near optimum at 34°C., and this temperature is sufficiently low so that inactivation of enzyme through prolonged exposure to the higher temperatures is prevented. If other substances, as inhibitors, are to be added, variations in the foregoing procedure may be made in order to

maintain the total volume of liquid at 3 cc. exclusive of the KOH used to absorb liberated CO_2.

COMPARISON OF RESPIRATION OF RHIZOBIUM TRIFOLII ON DIFFERENT SUBSTRATES

Application of the methods developed was made through a study of the rate of oxidation and the rate of dehydrogenation of methylene blue by *R. trifolii* on different types of substrates. Comparison of the results was facilitated by calculation of these on a relative basis with glucose as the standard, equal to 100. The data are summarized in table 4. The chief conclusions drawn from this study are:

1. General considerations. The relative rate of reduction of methylene blue by the organism in the presence of a given substrate is in general definitely lower than is the rate of oxidation of the same substrate. The *rank*, however, of the substrates as hydrogen donators shows good agreement whether molecular oxygen or methylene blue is taken as the hydrogen acceptor. Exceptions to this generalization are: arabinose and galactose are excellent hydrogen donators to O_2 but only fair to methylene blue; the reverse is true of formate; acetate is a very poor donator to methylene blue but is fair to good with oxygen.

2. Carbohydrates. No carbohydrate examined was as good a hydrogen donator as is glucose with the possible exception of arabinose. Glucose, fructose, galactose, mannose, arabinose, sucrose and maltose are good to excellent hydrogen donators to O_2 in the presence of *R. trifolii*; xylose, lactose and cellibiose are fair; rhamnose and raffinose are poor.

3. Polyhydric alcohols. The polyhydric alcohols were oxidized by O_2 in an unusual manner. The initial rate of respiration, which was rather low, increased with time; with carbohydrates the rate of respiration is constant. The rates for the first and last 45 minutes of the 2 to 3 hours' tests are given in the table. The initial lag suggests the formation of some intermediate, possibly the corresponding aldose, which provides a better substrate for respiration. The consistency of the results, independent of the number of carbon atoms in the polyhydric

TABLE 4

Relative rates of dehydrogenation of different sources of carbon by suspensions of R. trifolii using oxygen and methylene blue as hydrogen acceptors

SUBSTRATE	SUSPENSION 6	SUSPENSION 7		SUSPENSION H		AVERAGE	
	M.B.	M.B.	O_2	O_2	O_2	M.B.	O_2
I. Carbohydrate							
Glucose	100	100	100	100	100	100	100
Fructose	69	55	86	76	79	62	72
Galactose	51	56	87	98	96	54	94
Mannose		65	100			65	100
Xylose	53	64	67	63	66	58	65
Arabinose	57	63	116	118	104	60	112
Sucrose		62	85	90	85	62	87
Maltose	61	72	80		81	66	81
Lactose	28	28	51	62	66	28	60
Rhamnose				29	21		25
Cellibiose				73	67		70
Raffinose				33	18		25
II. Alcohols							
Glycerol					47 73		47 73
Erythritol	17	16	54 66	29 53	28 60	17	37 60
Sorbitol	49	60	77 90			55	77 90
Mannitol	31	29	56 94	32 77	38 79	30	42 83
III. Acids							
Formate	85	95	41	38	22	90	34
Acetate	19	*0*	51 81	42 67	40 64	10	44 71
Lactate	83	76	87	83	50	80	73
Pyruvate	32	37	72	76	74	35	74
Succinate	95	87	183	147	140	91	157
Malate	90	90	108			90	108
Fumarate	113	96	173	130	122	105	142
Maleate				27	25		26
Citrate	0	0	27	24	15	0	22
Tartrate	0	0	27	20	12	0	20
Oxalate				19	15		17

When two values are indicated, the first is the initial rate of respiration, the second is the final. All other values indicate that rate of respiration was constant throughout test.

alcohol, indicates a common mechanism for oxidation of this type of compound. With O_2 as the hydrogen acceptor there was little difference in the activity of these compounds; with methylene blue all were poor with the possible exception of sorbitol.

4. Acids. The oustanding feature of the studies on substrates was the rapid utilization of the four carbon dicarboxylic acids. With O_2 as the acceptor, the highest rates of respiration observed with any substrate were obtained with fumarate and succinate. Malate, although not quite as active as these two, was also an excellent hydrogen donator to O_2. With methylene blue these compounds were not so outstanding as hydrogen donators but were superior to the majority of substrates tested. Since these three acids together with oxaloacetic acid act as catalysts in respiration of certain tissues (Annau, *et al.*, 1935; Laki, Straub, and Szent-Györgi, 1937), and since oxaloacetic acid may be concerned with the symbiotic nitrogen fixation process (Virtanen and Laine, 1937), respiration of the rhizobia with these acids as substrates is being investigated in detail.

Respiration on formate was very poor and fell off rapidly with time, but this compound was one of the best donators to methylene blue. In contrast, acetate was poor to inactive with methylene blue as the acceptor but fairly active with O_2. Oxygen uptake with acetate was of the same type as with the polyhydric alcohols, viz., increase in the rate with time suggestive of the formation of an intermediate which is more readily attacked. Lactate and pyruvate were both fairly good hydrogen donators to O_2, but pyruvate was poor with methylene blue as the acceptor. It is likely that lactate is readily dehydrogenated to pyruvate in the presence of methylene blue, but further activation of hydrogen proceeds much more slowly. Citrate, tartrate, maleate, and oxalate were very poor hydrogen donators to O_2 and inactive toward methylene blue.

SUMMARY

The preparation of a suspension of "resting cells" of *Rhizobium trifolii* which has suitable characteristics for study of the respiratory activities of this organism is described.

The influence of storage of the cells, dilution of the suspension, addition of the specific stimulatory factor ("Coenzyme R"), concentration of buffer salts, pH, temperature and concentration of substrate on the respiration of *R. trifolii* has been determined. From these investigations has been developed a suitable technique for the study of the respiratory characteristics of this organism.

Comparison of the relative activity of a number of carbohydrates, polyhydric alcohols and organic acids as hydrogen donators when activated by *R. trifolii* with O_2 or methylene blue as acceptors has been made.

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REFERENCES

Allison, F. E., and Hoover, S. R. 1934 An accessory factor for legume nodule bacteria. Sources and activity. Jour. Bact., 27, 561–581.

Annau, E., Banga, I., Gözsy, B., St. Huszak, Laki, K., Straub, B., and Szent-Györgyi, A. 1935 Über die Bedeutung der Fumarsäure für die tierische Gewebsatmung. Ztschr. Physiol. Chem., 236, 1–68.

Burk, D. 1934 Azotase and Nitrogenase in *Azotobacter*. Ergebnisse Enzymforsch., 3, 23–56.

Burk, D., Lineweaver, H., and Horner, C. K. 1934 The specific influence of acidity on the mechanism of nitrogen fixation by *Azotobacter*. Jour. Bact., 27, 325–340.

Clifton, C. E. 1937 On the possibility of preventing assimilation in respiring cells. Enzymolgia, 4, 246–253.

Cook, R. P., and Stephenson, M. 1928 Bacterial oxidations by molecular oxygen. Biochem. Jour., 22, 1368–1386.

Crozier, W. J. 1924 On biological oxidations as a function of temperature. Jour. Gen. Physiol., 7, 189–216.

Dixon, M. 1934 Manometric Methods. Cambridge University Press.

Fred, E. B., Baldwin, I. L., and McCoy, E. 1932 Root nodule bacteria and leguminous plants. Univ. of Wis. Studies in Sci., 5, 343 pp.

Georgi, C. E., and Wilson, P. W. 1933 The influence of the tension of oxygen on the respiration of rhizobia. Arch. Mikrobiol., 4, 543–564.

Hoover, S. R., and Allison, F. E. 1935 A growth and respiration factor for certain rhizobia. 3rd Internatl. Cong. Soil Sci. Trans., 1, 158–160.

Kendall, A. I., and Ishikawa, M. 1929 The significance of certain reactions induced by "resting bacteria." Jour. Infect. Diseases, 44, 282–291

(and succeeding articles by Kendall and collaborators in the same journal).

Konishi, K., and Tsuge, T. 1934 On the respiration of nodule bacteria. Jour. Sci. Soil and Manure (Japan), 8, 297–308.

Laki, K., Straub, F. B., and Szent-Györgi, A. 1937 Respiratory catalysis for four-carbon dicarboxylic acids. Ztschr. Physiol. Chem., 247, I–II.

Lineweaver, H., Burk, D., and Horner, C. K. 1932 The temperature characteristic of respiration of *Azotobacter*. Jour. Gen. Physiol., 15, 497–505.

Neal, O. R., and Walker, R. H. 1935 Physiological studies on *Rhizobium*. IV. Utilization of carbonaceous materials. Jour. Bact., 30, 173–187.

Neal, O. R., and Walker, R. H. 1936 Physiological studies on *Rhizobium*. V. The extent of oxidation of carbonaceous materials. Jour. Bact., 32, 183–194.

Quastel, J. H. 1928 The study of "resting" or non-proliferating cells. Jour. Hyg., 28, 139–146.

Quastel, J. H. 1932 Bacterial enzyme reactions. Ergebnisse Enzymforsch., 1, 209–230.

Quastel, J. H. 1937 Enzyme formation in bacteria. Enzymologia, 2, 37–42.

Stephenson, M. 1930 Bacterial metabolism. Longmans, Green and Co., London, 320 pp.

Stephenson, M. 1937 Formic hydrogenlyase. Ergebnisse Enzymforsch., 6, 111–138.

Stephenson, M., and Gale, E. F. 1937 The adaptability of glucozymase and galactozymase in *Bacterium coli*. Biochem. Jour., 31, 1311–1315.

Stephenson, M., and Stickland, L. H. 1931 Hydrogenase: a bacterial enzyme activating molecular hydrogen. I. The properties of the enzyme. Biochem. Jour., 25, 203–214.

Stephenson, M., and Yudkin, J. 1936 Galactozymase considered as an adaptive enzyme. Biochem. Jour., 30, 506–514.

Stickland, L. H. 1934 Studies in the metabolism of the strict anaerobes. I. The chemical reactions by which *Cl. sporogenes* obtains its energy. Biochem. Jour., 28, 1746–1759.

Stone, R. W., and Werkman, C. H. 1937 The occurrence of phosphoglyceric acid in the bacterial dissimilation of glucose. Biochem. Jour., 31, 1516–1523.

Thorne, D. W., Neal, O. R., and Walker, R. H. 1936 Physiological studies on *Rhizobium*. VIII. The respiratory quotient. Arch. Mikrobiol., 7, 477–487.

Thorne, D. W., and Walker, R. H. 1935 Physiological studies on *Rhizobium*. III. Respiration and growth as influenced by the reaction of the medium. Jour. Bact., 30, 33–42.

Thorne, D. W., and Walker, R. H. 1936a Physiological studies on *Rhizobium*. VI. Accessory factors. Soil Sci., 42, 231–240.

Thorne, D. W., and Walker, R. H. 1936b Physiological studies on *Rhizobium* VII. Some physiological effects of accessory growth factors. Soil Sci., 42, 301–310.

Umbreit, W. W., and Bond, V. S. 1936 Analysis of plant tissue. Indus. and Engin. Chem., 8, 276–278.

Virtanen, A. I., and v. Hausen, S. 1931 Über die Tätigkeit der Leguminosebakterien und die Ausnutzung des in Wurzelknöllchen der Luguminosen gebundenen Stickstoffs durch Nicht-Leguminosen. Ztschr. Pflanzenernähr. A, 21, 57-69.

Virtanen, A. I., and Laine, T. 1937 Nitrogen fixation by excised nodules. Suomen Kemistilehti B., 10, 24.

Walker, R. H., Anderson, D. A., and Brown, P. E. 1934a Physiological studies on *Rhizobium*. I. The effect of nitrogen source on oxygen consumption by *Rhizobium leguminosarum* Frank. Soil Sci., 37, 387-401.

Walker, R. H., Anderson, D. A., and Brown, P. E. 1934b Physiological studies on *Rhizobium*. II. The effect of nitrogen source on oxygen consumption by *Rh. meliloti*, *Rh. trifolii* and *Rh. phaseoli*. Soil Sci., 38, 207-217.

Werkman, C. H. 1927 Vitamin effects in the physiology of microörganisms. Jour. Bact., 14, 335-347.

Wilson, P. W., and Fred, E. B. 1937 Mechanism of symbiotic nitrogen fixation. II. The pO_2 function. Natl. Acad. Sci. Proc., 23, 503-508.

Wood, H. G., Stone, R. W., and Werkman, C. H. 1937 The intermediate metabolism of propionic acid bacteria. Biochem. Jour., 31, 349-359.

Woods, D. D. 1936 Hydrogenlyases. IV. The synthesis of formic acid by bacteria. Biochem. Jour., 30, 515-527.

LIGHT AS A FACTOR IN THE PRODUCTION OF PIGMENT BY CERTAIN BACTERIA

JAMES A. BAKER

Department of Pathology and Bacteriology, New York State Veterinary College, Cornell University, Ithaca, New York

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Pigment formation is a phenomenon common in bacteria. Some workers have attempted to show that pigmentation serves a useful purpose, but, except for the purple bacteria, there is little evidence to indicate that pigments are really of value to the organisms that possess them. The bacteriologist makes use of them, it is true, for purposes of identification, but this can hardly be said to serve the organism.

Pigments are often variable and not infrequently the ability to form pigment is permanently lost without altering other metabolic activities in any noticeable way. That the presence of molecular oxygen is necessary for pigment formation has long been known. Obligatory anaerobes, therefore, are never pigmented. It is also well known that high incubation temperatures are unfavorable for pigment formation, and that continuous incubation at body temperature will often permanently deprive many of the saprophytic chromogens of their color-forming habits. The pH of the culture medium has an influence in many cases and also the presence of certain chemical substances in the culture medium.

Recently, while studying a group of acid-fast bacteria, an organism was observed which developed a rich pigment when grown in the presence of light but which was wholly unpigmented when incubated in darkness. The examination of a rather large collection of acid-fast organisms, most of which were pigment-forming, revealed an additional strain exhibiting this peculiar action toward light.

This experience was wholly new to every one in our laboratory, and a search of textbooks and manuals failed to unearth any reference to such a phenomenon. Finally, however, a reference (Prove, 1887) was found to an early observation of this sort.[1] Since this observation apparently has been overlooked by modern bacteriologists, we considered it desirable to call attention to it, and to describe the phenomenon as we have seen it in our cultures.

EXPERIMENTAL

The phenomenon was first observed in an acid-fast organism pathogenic for the small tropical killifish (*Platypoecilus maculatus*) with which we are working. This organism grows quite slowly. Its optimum temperature is about 30°C.; it does not grow at 37°C. From ten days to two weeks' incubation is required for fairly luxuriant growth, but the maximum growth is not attained until considerably later. Cultures which were incubated in the dark were essentially colorless, whereas those that were allowed to stand on the laboratory table in the presence of light developed a deep orange color. The work that is described below was done with this strain. The only other strain in our collection which exhibited the property was one which had been isolated from cow's milk by Plum.[2] This culture is quite unlike the killifish strain in all respects except the behavior toward light. It grows at 37°C. and develops quite rapidly. When grown in the dark it is unpigmented. In the light it takes on a rosy pink shade.

EXPOSURE TO DIFFERENT LIGHT RAYS

In these brief studies, no attempt was made to measure the quality or the intensity of the light sources used. Three kinds

[1] *Micrococcus ochroleucus* found in the urine of man.

Morphology—micrococcus from 0.5–0.8 microns in diameter, solitary, in pairs, or in short chains.

Biological characters—aerobic, non-liquefying, chromogenic micrococcus. Develops in usual culture media at room temperature. Colonies appear on gelatin plates at the end of twenty-four hours. From the description this organism apparently does not belong to the acid-fast group of bacilli.

[2] Culture received in 1932 from N. Plum, Serum Laboratory, Royal Veterinary and Agricultural College, Copenhagen, Denmark.

of light were tested for their efficiency in producing coloration in fully developed cultures which had been grown in the dark—sunlight, light from a 100-watt Mazda lamp, and ultra-violet light from a small, water-cooled, mercury-vapor lamp equipped with a Wood filter.

The sunlight exposures were made in the middle of the day during August and September. The exposures to the incandescent lamp were made at a distance of approximately two feet. Exposures to the mercury vapor lamp were at a distance of about three inches.

TABLE 1

The effects of light on pigmentation

LIGHT SOURCE	TIME EXPOSED	RESULT
Sunlight	1 minute	Partially colored
Sunlight	15 minutes	Full coloration
Sunlight	60 minutes	Partially colored
Sunlight	100 minutes	No pigmentation
Incandescent lamp	5 minutes	No pigmentation
Incandescent lamp	15 minutes	Partially colored
Incandescent lamp	30 minutes	Full coloration
Ultra violet	10 seconds	Partially colored
Ultra violet	1 minute	Full coloration
Ultra violet	20 minutes	No pigmentation

Exposures to the incandescent lamp and to sunlight were made in tubes (glycerol-agar slants). Cultures exposed to the ultra-violet lamp were growing on agar plates, from which the lids were removed during the exposure period.

The appearance of pigment does not occur immediately upon exposure to light. Usually coloration can be seen within twenty-four hours after exposure is begun and virtually the full intensity is reached within forty-eight hours. The results which are shown in the following table were recorded after forty-eight hours' incubation in darkness following the exposure period.

These data suggest that it is the shorter light rays that affect pigmentation. Although direct comparisons can not be made

because of the differing intensities of the light sources, it is significant that the ultra-violet operated very quickly, and that sunlight was much more effective than light from a Mazda lamp. It will be noticed that exposure to ultra-violet for 20 minutes and to sunlight for 100 minutes produced no pigmentation. Evidently these exposures were lethal; at any rate subcultures could not be obtained.

TABLE 2

The temperature influence on pigmentation

EXPOSURE TEMPERATURE	STORAGE TEMPERATURE	RESULT
°C.	°C.	
4	4	No pigment
	24	Partial (+)
	37	Partial (++)
24	4	No pigment
	24	Partial (++)
	37	Partial (+++)
37	4	No pigment
	24	Full color (++++)
	37	Full color (++++)
Unexposed controls	4	No pigment
	24	No pigment
	37	No pigment

EFFECTS OF TEMPERATURE ON PIGMENT FORMATION

Experiments were conducted to test the influence of temperature on the formation of pigment. Three temperatures, 4°, 24°, and 37°C. were employed. The cultures were on agar slants, and were fully-grown and non-pigmented in the beginning. The culture tubes were placed in glass beakers filled with water which was maintained at the desired temperature. All beakers were freely exposed to bright, midday sunshine for thirty minutes. After exposure the cultures were grouped into three lots which were then held in the dark at 4°, 24°, and 37°C. respectively. The degree of pigmentation in each case is indicated in table 2.

Thirty-seven degrees Centigrade, a temperature at which the

organism will not grow, favored chromogenesis more than 24°C., a temperature at which growth will develop. Color formation did not take place when the cultures were stored at 4°C. Further observations have shown that when the cultures stored at 4°C. were subsequently placed at 37°C. for 48 hours, the same degree of pigmentation developed as in those stored at 37°C. immediately after exposure to sunlight. Pigmentation did not occur in the control cultures.

OXYGEN RELATIONSHIP TO PIGMENT FORMATION

Wright's method of chemical absorption of oxygen with alkaline pyrogallol was used to remove the free oxygen from fully developed, non-pigmented cultures. These cultures, as well as aerobic cultures of the same age and grown on the same medium, were then exposed to sunlight for 30 minutes. Pigment did not develop in the anaeorobic tubes. The anaerobic environment apparently did not harm the bacteria and subsequent exposure under aerobic conditions showed the usual pigmentation. The control tubes, having access to atmospheric oxygen, showed color formation. Molecular oxygen was, therefore, apparently necessary for pigment formation.

THE LIVING ORGANISM AND PIGMENT FORMATION

We have not attempted an exhaustive study of the mechanism of pigment formation by this organism under the influence of light. We wondered whether light acted simply to activate a chemical change which conferred color on some substance which had already been elaborated in an uncolored state, or whether the color-containing substance was formed only under the influence of light.

Three observations, however, tend to indicate that light so alters the metabolic activities as to cause the organism to form pigment, and that a precursor of this pigment is not present in cultures incubated in darkness. These observations are:

1. Unpigmented cultures killed by heating at 60°C. for thirty minutes will not produce pigment.

2. Unpigmented cultures exposed to sunlight or ultra-violet

light long enough to destroy their vitality do not thereafter produce pigment.

3. The pigment of this organism is readily soluble in alcohol. When unpigmented cultures are extracted with alcohol, however, and the extract is exposed to light while being constantly aerated, no pigment appears in the solution.

The fact that pigment forms rather rapidly after light exposure in fully grown cultures, which are colorless as a result of having been grown in darkness, is an indication that the coloring substance appears in old cells. This is also indicated by its formation at 37°C., a temperature which is too high for multiplication but not high enough to destroy the vitality of the culture.

SUMMARY

1. Two cultures of acid-fast bacteria have been found which form pigment when cultivated in the presence of light and are wholly devoid of it when cultivated in darkness.

2. Brief exposure to ultra-violet and sunlight, and longer exposures to electric light, confers on fully developed unpigmented cultures the ability to form pigment during a subsequent period in darkness.

3. The mechanism of pigment formation under the influence of light is not known; however, it is evident that it is a vital phenomenon and is not merely the result of a chemical reaction induced by light in substances preformed by the organism. The evidence for this statement is as follows: (*a*) Unpigmented organisms killed by heat or by ultra-violet light do not develop pigment. (*b*) Pigment is not formed in living cultures incubated at 4°C. after exposure. (*c*) Whereas, alcohol will extract the pigment from cultures grown in the presence of light, alcoholic extracts of non-pigmented cultures do not become colored when so exposed.

The writer wishes to acknowledge his indebtedness to Dr. W. A. Hagan for the many helpful suggestions in carrying out this work.

ADDENDUM

Since this paper was submitted for publication it has been discovered that the phenomenon described is not a rare one. Of a lot of 185 strains of acid-fast bacilli, 24 strains have been found in which light affects pigment formation. Of these, 22 produced slight pigment when grown in the dark and brilliant orange when grown in diffuse daylight. Two other strains were wholly colorless when cultivated in darkness and pigmented when exposed to light. One of the latter was a strain of the avian type of tubercle bacillus. Although cultures of the mammalian tubercle bacillus have not been tested for this specific reaction, it has been noticed that strains handled by students in class work often exhibit considerable pigmentation, whereas the same strains kept in our stock collection do not exhibit color. It is thought that exposure to light may account for this difference.

REFERENCE

PROVE, O. 1887 *Micrococcus ochroleucus*, eine neue chromogene Spaltpilzform. Beit. z. biol. Pfl., 4, 409–439.

THE EFFECT OF SALTS UPON THE GERMICIDAL ACTION OF PHENOL AND SEC-AMYLTRICRESOL[1]

H. W. LUNDY

Massachusetts Institute of Technology, Cambridge, Mass.

Received for publication November 30, 1937

Very little work has been reported in the literature concerning the effect of salts upon the phenol coefficient. Neither the action itself nor the mechanism through which the effect is produced has been studied sufficiently to justify definite conclusions in regard to this phase of disinfection.

While the action of salts, acids and alkalies on bacteria has been studied extensively, their effect on various disinfectants has been neglected. It has been assumed, as Eisenberg and Okalska concluded in 1913, that the more toxic a salt is by itself the more it will increase the germicidal activity of the disinfectant. That this assumption is not always correct will be shown in this paper. Römer (1898) concluded that the greater the salt concentration of a disinfectant solution the greater the increase in the disinfectant action. This also will be shown not to hold true for all salts.

The present work was undertaken to test the action of various salts when added to solutions of phenol and sec-amyltricresol and to study the manner in which these salts produce their effect. The sec-amyltricresol was obtained from the Upjohn Company. Most of the salts were Baker's C. P. chemicals.

EXPERIMENTAL PROCEDURE

The technique used was essentially that described in the United States Department of Agriculture Circular Number 198. The salt to be tested was accurately weighed out and placed in a volu-

[1] Contribution No. 121 from the Department of Biology and Public Health, Massachusetts Institute of Technology. The work was supported by a fellowship from the Upjohn Company.

metric flask. 5 per cent phenol or 1 per cent sec-amyltricresol was then added to the flask and this solution was used as the unknown disinfectant, final dilutions being made in the medication tubes with distilled water. In these experiments in which a salt was added to a disinfectant a phrase comparable to that of "phenol coefficient" was needed to express the results numerically. Therefore the term "disinfectant coefficient" was coined. This was defined as the ratio of the greatest dilution of the disinfectant plus salt killing the test organism in ten minutes but not in five, to the greatest dilution of the disinfectant alone showing the same results. This is a general term and may be given specific meaning by listing the disinfectant, salt and test organism used. It is obvious that if the salt has no effect the coefficient will be one.

For the work with phenol a five per cent stock solution was prepared and dilutions made from this as needed. For the stock solution of sec-amyltricresol the following tincture was prepared: sec-amyltricresol 1 gram, pure ethyl alcohol 50 cc., acetone 10 cc., water, q.s. 100 cc. Further dilutions were prepared daily as needed.

For those experiments in which pH measurements were desired, sufficient amounts of each dilution were prepared so as to permit determinations to be made on one portion before the disinfectant coefficient test was run and still leave 5 cc. in each medication tube for the test itself. Determinations were also made at the end of the test to discover the actual pH values at which the disinfectants were acting.

Since pH measurements on every tube in the test would be impractical if a wide range of salts were to be used, it was decided to limit the determinations to the first and the last dilution of each test. There were usually seven medication tubes containing the salt and this left five intermediate tubes for which the pH value had to be interpolated. Tests in which the pH determinations were made upon every tube showed that the intermediary values could be interpolated quite closely so that the error introduced by this procedure was not greater than others inherent in the technique of the test itself.

The test organisms used were *Staphylococcus aureus*, Rosenbach

(Government strain No. 209) and *Escherichia coli* (Migula) Castellani and Chalmers, both of which were obtained from the stock collection of the Department of Biology and Public Health at the Massachusetts Institute of Technology. The medication temperature for all tests was 37 degrees Centigrade.

Phenol (test organism, Staph. aureus)

In concentrations of both 2 and 10 per cent, NaCl, LiCl, NH_4Cl, $BaCl_2$, $FeCl_2$, Na_2SO_4, $FeSO_4$, Na_2HPO_4, KH_2PO_4 and Na_3PO_4 showed hardly any enhancement of the disinfectant coefficient when *Staphylococcus aureus* was used as the test organism. The highest coefficient obtained with these salts was 1.4 with 10 per

TABLE 1
Effect of pH on phenol

pH BEFORE ADDING CULTURE	DISINFECTANT COEFFICIENT	pH AFTER EXPERIMENT
2.6	1.4	4.6
2.8	1.5	6.0
6.0	1.0	7.2
7.0	1.0	7.2
8.8	0.82	8.7
8.9	1.0 or less	8.8
9.0	0.94	8.8
9.1	0.94	9.0
9.2	<0.93	9.0

cent $BaCl_2$. Ten per cent Na_3PO_4, which produced a markedly alkaline reaction, reduced the coefficient to 0.61.

$FeCl_3$, $Fe_2(SO_4)_3$ and $CuSO_4$, in the same concentrations as with the above salts, produced coefficients ranging from 1.9 to 3.3. Both HCl and H_2SO_4, when added to phenol to give approximately the same pH as a 2 per cent concentration of $Fe_2(SO_4)_3$ in aqueous solution, produced a coefficient of 2.0 or more. Two per cent $FeCl_3$ and 2 per cent $Fe_2(SO_4)_3$ in aqueous solutions did not kill the test organism in 15 minutes even in a dilution of 1:2 so the enhancement cannot be explained on a basis of toxicity alone.

The above table shows the effect on the coefficient of the

addition of hydrochloric acid and sodium hydroxide to phenol. The pH values given are those of the dilution in which the test organism was killed in ten minutes but not in five, a Beckman glass electrode being used to measure the pH.

This table not only illustrates the decrease in germicidal ability of phenol as the pH increased but demonstrates the error that would be made if it were assumed that the pH value before adding the bacterial culture was that at which the disinfectant was actually acting. A difference of 4.9 pH units was observed in one experiment. It is evident that the buffering action of the bacterial culture was not as effective in alkaline solutions as in acid ones.

TABLE 2

Effect of salts on Sec-amyltricresol

SALT	pH BEFORE ADDING CULTURE	DISINFECTANT COEFFICIENT	pH AFTER EXPERIMENT
2 per cent $FeCl_3$	2.4	28.9	2.4
2 per cent $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4$	2.4	44.4	2.8
2 per cent $Fe_2(SO_4)_3$	2.6	103.8	3.2
0.5 per cent $Fe_2(SO_4)_3$	2.6	32.2	3.5
0.1 per cent $Fe_2(SO_4)_3$	2.7	8.9	3.6
2 per cent $K_2Cr_2O_7$	4.2	13.0	5.2
2 per cent $FeSO_4$	4.4	1.1	6.8
2 per cent $KMnO_4$	7.3	37.5	8.0

Sec-amyltricresol (test organism, Staph. aureus)

In a concentration of 2 per cent, NaCl, $BaCl_2$, $FeCl_2$, $FeCl_3$, $NaNO_2$, Na_3PO_4, $(NH_4)_2HPO_4$, $CoCl_2$ and $KMnO_4$ had practically no effect on the disinfectant coefficient. Two per cent $CuSO_4$ gave a coefficient of 1.9. Two per cent ferric alum, $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4$, produced a coefficient of 2.5. Using a 0.1 per cent tincture of sec-amyltricresol as a stock solution instead of a 1 per cent tincture the following disinfectant coefficients were obtained for $Fe_2(SO_4)_3$: 2 per cent = 20.0, 0.5 per cent = 5.3, 0.1 per cent = 1.4.

Sec-amyltricresol (test organism, Esch. coli)

Table 2 gives the average disinfectant coefficients found when various salts were added to sec-amyltricresol. The pH values

given are those of the dilutions in which the test organism was killed in ten minutes but not in five.

These figures indicate that it is not through a lowering of the pH alone that salts exert their action in enhancing the bactericidal ability of a disinfectant. Furthermore, a specificity of action is demonstrated toward *E. coli* as compared to *S. aureus* in the case of certain salts. Ferric chloride, ferric sulphate and ferric alum all produced greater enhancement of the disinfectant coefficient when *E. coli* was used than when *S. aureus* was the test organism. This is contrary to the general belief that Gram-positive organisms are more susceptible to the action of salts than are Gram-negative bacteria.

In this connection it was interesting to note, while working with the 0.1 per cent tincture of sec-amyltricresol, that the killing power toward *E. coli* (killing in ten minutes but not in five) of this strength solution was practically identical with that of a 1 per cent tincture. Both dilutions being high enough to rule out any bactericidal action of the solvents, it is difficult to understand why the former, which contained only one-tenth as much of this active disinfectant as the latter, should exert the same killing effect. Although this phenomenon does not hold true for *S. aureus*, further work will have to be done before it can be explained as one of the differences between Gram-negative and Gram-positive organisms.

TABLE 3

Effect of pH on Sec-amyltricresol

pH BEFORE ADDING CULTURE	DISINFECTANT COEFFICIENT	pH AFTER EXPERIMENT
6.8	1.0	7.5
9.5	1.1	7.7
10.1	1.2	8.2
10.7	1.2	8.9
10.9	1.7	9.3

A 2 per cent aqueous solution of $Fe_2(SO_4)_3$ does not kill *E. coli* in a dilution of 1:10 in the 15-minute period used in the test so that the enhancement is not due to an inhibition of the organism. This fact was also indicated by making secondary sub-

cultures with four loopfulls of broth from the first subculture tubes. No inhibition of growth was observed.

Table 3 shows the effect of NaOH upon sec-amyltricresol. The pH values given are those of the dilution in which the test organism was killed in ten minutes but not in five.

Here, the disinfectant coefficient increased as NaOH was added to sec-amyltricresol and *E. coli* used as the test organism. When NaOH was added to phenol and *S. aureus* was used as the test organism the disinfectant coefficient decreased as the pH increased.

When a salt in a concentration of 2 per cent was added to sec-amyltricresol as in the above experiments, the original pH was maintained much better upon adding the bacterial culture than when HCl was used even though the pH was the same to begin with in each case.

Some preliminary work with the oxidation-reduction potential electrode showed that, in general, salts which produced high Eh values likewise produced high disinfectant coefficients. However, there was found no direct relationship and further work will have to be done before definite conclusions can be drawn.

SUMMARY AND CONCLUSIONS

When added to phenol in concentrations of 2 and 10 per cent many salts showed no significant enhancement of the disinfectant coefficient[2] when *Staphylococcus aureus* was used as the test organism. In general, bi-valent salts produced greater effect than did uni-valent ones and tri-valent salts greater than bi-valent ones.

Very similar results were obtained when salts were added to sec-amyltricresol. $CuSO_4$, $Fe_2(SO_4)_3$ and $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4$ gave higher disinfectant coefficients than did the other salts tested. Since these salts were non-toxic by themselves and yet produced such marked increases in the disinfectant coefficient it would seem that toxicity *per se* is not an index to the degree which a salt will enhance the germicidal action of a disinfectant.

[2] Defined as the ratio of the greatest dilution of the disinfectant plus salt killing the test organism in ten minutes but not in five, to the greatest dilution of the disinfectant alone showing the same results.

The concentration of a salt is likewise not a measure of the degree of enhancement since many salts showed no greater increase in the disinfectant coefficient in a 10 per cent than in a 2 per cent solution.

Although when HCl and H_2SO_4 were added to phenol a similar increase in the disinfectant coefficient was obtained, experiments showed that it was not through a lowering of the pH alone that salts exert their enhancing action. A specificity of action was demonstrated with certain salts towards *Escherichia coli* as compared to *Staphylococcus aureus*, the coefficient being much higher with the former organism.

Preliminary oxidation-reduction potential measurements indicated that Eh may also be a factor involved in the ability of salts to increase the killing power of disinfectants.

REFERENCES

Eisenberg, P., and Okolska, M. 1913 Untersuchungen zur theorie der desinfektion. Centrabl. f. Bakt. Abt. I, 69, 312–346.

Römer, C. 1898 Ueber desinfection von milzbrandsporen durch phenol in verbindung mit salzen. Munch. med. Wschr., 45, 298–302.

United States Food and Drug Administration 1931 Methods of Testing Antiseptics and Disinfectants. Dept. of Agr. Circular 198.

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN PENNSYLVANIA CHAPTER

PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, NOVEMBER 23, 1937

VIRUS ACTIVITIES IN RELATION TO CANCER. *Peyton Rous*, The Rockefeller Institute for Medical Research, New York City, N. Y.

Causative agents with the character of viruses have been obtained from many chicken tumors and also from the cutaneous papillomas of western cotton-tail rabbits. The papillomas occasionally become carcinomatous in their natural host, and those produced by the inoculation of domestic rabbits with the virus frequently do so, the cancers arising from the cells infected with the virus. Under such circumstances the latter serves as a potent "*carcinogenic agent*", one, that is to say, which brings on cancer. The virus responsible for condyloma acuminatum of man sometimes acts in the same way. In skin that has been altered by repeated tarring, the rabbit virus elicits cancers forthwith as well as papillomas. Sometimes they are the outcome of infection of preexisting tar warts which otherwise would disappear. In such instances the virus acts as the *effective cause* of the cancers. Strong evidence exists that it functions as the *real cause* of other cancers that it elicits in the tarred skin. This evidence was reviewed.

EASTERN PENNSYLVANIA CHAPTER

ONE HUNDRED AND TWENTY-EIGHTH MEETING, PHILADELPHIA COUNTY MEDICAL SOCIETY BUILDING, JANUARY 25, 1938

NOTES ON STEAM PRESSURE STERILIZATION. *Carl J. Bucher*, Jefferson Hospital, Philadelphia, Penna.

The largest surgical packs of dry goods, fluids and other materials were placed in the coolest part of a steam pressure sterilizer and sterilization carried out under varying conditions of time, pressure, volume, manner of loading the sterilizer, etc. Temperatures at the center of the volume of different materials, were recorded graphically at three-minute intervals by means of a thermocouple. The time required to kill the dried spores of *Bacillus subtilis* at the center of such packs was also determined. It was concluded that the unqualified statement made in text-books of bacteriology, that steam pressure sterilization performed for 20 to 30 minutes at 15 pounds pressure or 221°C. will kill all microbic life, is misleading. To perform efficiently steam sterilization, many factors must be taken into consideration. Temperature and not pressure is the chief criterion of control. The time necessary for sterilization varies with volume, density and

physical state of the article to be sterilized, the load in the sterilizer, the nature of the material to be sterilized, the manner of loading the instrument, the position of certain articles in the sterilizer, and the mechanical structure of different types of pressure sterilizers.

EASTERN PENNSYLVANIA CHAPTER

One Hundred and Twenty-ninth Meeting, Philadelphia County Medical Society Building, February 22, 1938

Bacteriophagic Service in Septic Conditions. *Ward J. MacNeal*, Department of Pathology and Bacteriology, New York Post-Graduate Medical School and Hospital, Columbia University, New York City.

In a large series of cases, in which bacteriophage therapy was tried as a last resort, there appeared to be grounds for assuming that the treatment was efficacious. The mechanism of the bacteriophage action in the body seemed to be the stimulation of phagocytosis. The majority of cases were infections due to staphylococci. One distinct advantage of bacteriophage treatment is that other forms of treatment are not contraindicated but may be carried on at the same time.

EASTERN PENNSYLVANIA CHAPTER

Nineteenth Annual March Meeting, Philadelphia County Medical Society Building, March 22, 1938

Bacteriological Studies on Rheumatic Fever Cases (680 cases). *Rose Ichelson*, 726 S. 59th Street, Philadelphia.

In the study of 680 cases of rheumatic fever, cultures from nose and throat showed the following organisms present in pure culture: *Streptococcus viridans*, 102; *Streptococcus hemolyticus*, 81; *Streptococcus non-hemolyticus*, 54; *Staphylococcus aureus*, 40 and *Klebsiella friedländeri*, 20 cases. The remaining cases showed mixed cultures of the above organisms.

From stools of the 680 cases, 160 showed *Streptococcus viridans* and 90, *Streptococcus non-hemolyticus*.

Each patient was skin tested to all organisms and their mixed exotoxins. Positive reactions were obtained as follows: saline controls, 0; bouillon controls, 6%; *Streptococcus viridans*, 32%; *Streptococcus non-hemolyticus*, 6%; *Streptococcus hemolyticus*, 4%; *Staphylococcus aureus* (hemolytic), 38%; *Staphylococcus aureus* (non-hemolytic), 8%; *Staphylococcus albus*, 1%; *Klebsiella friedländeri*, 4%; *Neisseria catarrhalis*, 2%; unidentified Gram negative bacilli, 2%; *Escherichia coli*, 3% and their mixed toxins, 90%.

On 50 cases where blood cultures were taken, 3 showed non-hemolytic streptococci. Vaccines and exotoxins were prepared from those organisms to which the patients were sensitive. Clinical findings in reference to this study will be reported in the near future by Drs. J. B. Wolffe and Victor A. Digilio, who referred the cases.

Demonstrations of the Methods for Measuring Sanitary Ventilation. *William F. Wells* and *Ruth Blumfeld*, Laboratories for the study of air-borne infection, University of Pennsylvania, School of Medicine, Philadelphia, Pa.

The authors demonstrated the apparatus and technic by which sanitary

ventilation is measured by actually testing the Auditorium of the Philadelphia County Medical Society during the meeting of the Eastern Pennsylvania Chapter.

Sanitary ventilation is defined as the rate at which microörganisms are vented, or as the proportional air replacement which would remove the equivalent number of microörganisms eliminated by any other means.

A test organism was thrown into the air by means of an atomizer, the "infector". The droplets evaporated instantly, leaving suspended in the air droplet nuclei to drift about on the air currents. An air centrifuge, the "infectee", placed on the opposite side of the Auditorium, sampled the air to determine the bacterial concentration.

Two determinations of the sanitary ventilation of the Auditorium were made, one with and one without an ultra-violet burner which irradiated the air above the eye level. The method of counting the air centrifuge tubes was demonstrated and the method of computing the proportional pure air replacement which would bring about by any means a given reduction in microorganisms.

(This technic was reported in the March issue of the American Journal of Public Health.)

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